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The antimicrobial activity and essential oil composition of medicinal aromatic plants used in African traditional healing

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Chapter 1

Overview of the use of aromatic plants and their essential oils to treat microbial infections.

1.1 Introduction

There can be no doubt that antimicrobials are one of the most important therapeutic agents available today (Clark, 1996). The importance could most likely be attributed to the fact that most modern treatment regimens for ailments are effectively managed by pharmaceuticals. However, the treatment of infectious diseases focuses on a cure rather than symptomatic relief or merely pharmacological management. The impact of improved quality of life and increased life expectancy with chemotherapeutic agents has brought man-kind into an era of antibiotic dependency. Man's reliance on these antimicrobial agents has led to their global misuse. This has resulted in untreatable infections due to emerging antimicrobial resistance.

There are a number of international strategies in place to try and curb this surge of antimicrobial resistance. Infection control, antibody-based therapies and rational use of antibiotics are currently accompanied by the parallel development of new antimicrobial agents. Scientific advances have gone full circle and are presently probing plant derived antimicrobials to seek evidence corroborating the traditional use that has been holistically instilled into the cultural evolution of indigenous people. The importance and need for further research into traditional herbal medicine was first officially recognized by the World Health Organization (WHO) in 1978 (Kim, 2005). Subsequently, the WHO declared the period 2001-2010 as the decade for African Traditional Medicine. Initiatives were set up to prioritise medicinal plant screening with the aim to initiate an African Pharmacopoeia (AACHRD, 2002). In the WHO document for Traditional Medicine 2002-2005, a strategy was suggested to address reliability of methods, safety, quality, access and rational use. In a follow-up presentation in 2005, the global outcomes were presented (WHO, 2005). With the worldwide emphasis on traditional medicines, the basic screening and more in-depth research protocols into natural products cannot be over emphasised.

1.2 A historical perspective

Historically plants have provided a good source for the treatment against microbial infections. References to the therapeutic application of aromatic plants used for antimicrobial purposes are well documented in ancient history. Hieroglyphic depictions have been found (Figure 1.1 and Figure 1.2) of essential oils used for anointing the sick. Traces of frankincense oil, found in an urn were discovered in Tutankhamen's tomb. Their use in embalming processes suggests that the preservative nature of the oils were beneficial in warding off putrefaction. In ancient times, it has generally been the essential oils rather than extracts that were considered effective (Rios and Recio, 2005). Records, written on clay tablets dating back to about 2600 BC indicate that amongst others, oils from *Cedrus* (Cedar) and *Commiphora* (Myrrh) species were used for ailments ranging from coughs and colds to inflammation (Gurib-Fakim, 2006). The oldest written information from the Egyptians is the Ebers Papyrus. The origin dates back to around 1500 BC. The Ebers Papyrus is a medical "handbook" documenting therapeutic treatments and pharmaceutical plant preparations. It has been said that the diagnostic precision is very accurate (Heinrich *et al.*, 2004). Accounts of distillation by the Egyptians, Indians and Persians over 2000 years ago have been documented and the first authentic written account of essential oil distillation is ascribed to Villanova ca. 1235-1311 (Burt, 2004).



Figure 1.1



Figure 1.2

Figure 1.1 and Figure 1.2 Egyptian hieroglyphic depictions of essential oils used for anointing and medicinal purposes. **Figure 1.1** depicts an Egyptian with an incense burner preparing oils for inhalation (http://www.lotuspress.com/lotusbrands/tiferetonline/key.html). **Figure 1.2** illustrates the administration of oils for the enhancement of body processes (http://www.ansononline.com/cgi-bin/ez-catalog/cat_display.cgi?0X367200).

Biblical citations of medicinal plant use account for at least 30 references with myrrh and frankincense the most noteworthy (Cowan, 1999). The antimicrobial attributes of frankincense (*Boswellia* spp.) was especially favoured by the Greeks, Romans, Babylonians, Persians and Hebrews where it was used as a treatment for wounds, skin diseases, urinary tract infections and gynaecological disorders. Myrrh (*Commiphora* spp.) was used in embalming because of its effectiveness in preventing bacterial growth. It was also valued for its effectiveness in the treatment of topical conditions (Dharmananda, 2003; Marshall, 2004).

One of the earliest reports of the use of plant material for antimicrobial use was when the European "iceman" was first discovered. Traces of *Piptoporus betulinus*, a bracket fungus was found accompanied by the preserved human dating back to 3300 BC. Capasso (1998) postulated that the plant had antimycobacterial as well other microbial inhibitory activities.

Later, the Greeks and Romans played a significant role in documenting the use of medicinal plants. The Greek scholar Pedanius Dioscorides (1 BC) described more than 600 medicinal plants which became the prototype of modern pharmacopoeias (Cowan, 1999; Heinrich *et al*., 2004). Hippocrates, the well-known Greek medical doctor (460-375 BC) to whom the Hippocratic Oath is attributed, fully supported the use of herbal medicines and advocated their use.

After the conquest of the Roman Empire, Greek medical texts were translated to Arabic, and then later copied by the Christians. Printed European reports on medicinal plants through the 16th century were still very strongly influenced by the Greek-Roman sources. Herbal documentation became available in various languages and became the driving force of European phytotherapy.

During the middle ages aromatic plants were used extensively to combat the plague. Widespread use of perfumed candles, pomanders (plant material adorned the neck of wearers) and burning of oils were believed to offer some immunity to the caregivers of the sick. During this time, plant treatment regimens were greatly contradicted. On the one hand monks were respected for tending to the sick, using herbal medicine (Heinrich *et al*., 2004). On the other hand the village herbalists were persecuted for witchery.

During the renaissance period there was a revival of holistic treatments. It is about this time that the term "pharmacognosy" was born. The word is derived from the two Greek words

"*pharmakon*" meaning drug and "*gignosko*" meaning to acquire knowledge. The focus of pharmacognosy studies during this time and up to the 20th century were on medicinal substances derived from the oils, waxes, gums and resins of plants (Kinghorn, 2001). It was highly thought of as the mother of all present-day pharmaceutical disciplines (Gurib-Fakim, 2006). With the emergence of antibiotic discovery in 1939, plants as antimicrobials were overlooked in favour of more potent treatment regimens. With the introduction of antibacterial compounds such as penicillin and streptomycin, effective therapy against *Staphylococcus* and other infections were met (Rice, 2006). These discoveries led to the era of wonder drugs where the "single, golden bullet" approach to combat diseases were the first line of therapy.

1.3 Present use of antimicrobials and the impact on disease

Antimicrobials have been used successfully for over six decades. At least 30 new antimicrobial agents have been introduced into mainstream chemotherapeutic treatment regimens within the first 18 years of the antibiotic era. This has been marked by cycles, comprising of the introduction or development of an antibiotic followed by the subsequent emergence of resistance (Swartz, 2000). Given the current problem with the development of bacterial resistance there is a great surge to discover new potent antibiotics. The present world market for antibiotic drug expenditure on finding new anti-infective agents is expected to increase by 60% from spending levels in 1993. The continued interest in finding newer anti-infectives are exacerbated by emergence of resistance and its impact on the global population encompassing both developed countries and third world countries. Initially antimicrobial resistance was mostly evident in hospital environments where nosocomial infections became prevalent due to the consistent exposure to antimicrobials. More recently the spread of resistance has become community-acquired. The available epidemiological data suggests that levels of antimicrobial resistance have reached unacceptable proportions and trends show further increases (Okeke *et al.*, 2005). Infectious diseases still account for approximately half of all deaths in tropical countries (Iwu *et al.*, 1999). Microbial infections contribute towards the primary morbidity and mortality statistics from hospitals around the world (Rice, 2006). These mortality rates for developed countries are increasing in spite of adequate hygiene, sanitation and education. It has been estimated that as many as 30% of people in industrialized countries still suffer from diarrhoeal-related diseases (Kosek *et al.*, 2003).

It is also estimated that approximately 80% of the total global population comprise of developing nations. These developing nations often have poorer sanitation and hygiene levels which contribute to infection susceptibility, Even though the success of antibiotics has benefited mankind, many poorer countries have not had accessibility. The burden of infectious diseases falls heavier on developing countries where it has been estimated that approximately 60% of all deaths are related to communicable diseases. This is in sharp contrast to the 35% mortality from developed countries (Istŭriz and Carbon, 2000). Furthermore, third-world countries are subject to a shortage of funds, adequate medical care and lack the resources for newly developed medicine, which make them dependent on their natural resources.

The increase of numerous infectious diseases that have occurred on a global level have further emphasised the need to continue with antimicrobial studies (Cordell and Colvard, 2005). In addition, the recent limitations on the use of antibiotic feeds in farming and the call for the reduction of antimicrobial chemical additives in foods are now regulated more than ever before. Thus the search for natural antimicrobials has become increasingly popular (Devlieghere *et al*., 2004).

1.4 Ethnomedicinal plants as antimicrobials

Phytochemicals have found their way into the arsenal of antimicrobial drugs prescribed by physicians. The sale and use of herbal products worldwide has increased dramatically with Germany as the leading country where 80% of medical practitioners are prescribing herbal remedies (Gilani and Rahman, 2006). Natural products of botanical origin represent almost 40% of all drugs in clinical use. Phillipson (2001) estimated that over 50% of the top 20 drugs used on today's world market could be linked to natural product research. The modern pharmacopoeias contain at least 25% plant derived drugs (Kim, 2005). The primary benefit of sourcing plant material for medicines is that they are perceived to be safer, less rigorous and more affordable than synthetic alternatives (Iwu *et al.,* 1999). In spite of the successes of conventional antimicrobials sourced from microbial products, botanical substances are still researched for potential biological activity. Borris (1996) hypothesised that plants and micro-organisms are complementary, both systems are natural and have a diverse species base, however, each produce different metabolites and therefore research into both systems are necessary for antimicrobial screening.

Rios and Recio (2005) commented on the increase in published articles relating to medicinal plants for antimicrobial therapy. During 1966-1994, there were 115 articles found on Pubmed. In later years (1995-2004) the authors noted that the references more than doubled to 307. Where essential oils were researched, references increased to 323 in 2005 (Rios and Recio, 2005). A recent literature Pubmed search undertaken for "plants" and "antimicrobial" yielded 324 citations exclusively for 2006, in comparison with the earlier publications (256 articles in 2003). Similarly using Science Direct, only 44 papers were published in 2002 in comparison with the 84 papers published in 2006. These estimates confirm the exponential rise in antimicrobial ethnobotanical research. The rapid increase in research in this field is possibly due to the increased interest in overcoming antimicrobial resistance to conventional antimicrobials. In addition, improved assessment techniques within pharmacognosy as well as the diversity of naturally occurring metabolites allows for a number of studies of this nature (Clark, 1996).

1.5 Essential oils as antimicrobials

Many essential oils extracted from plants are known for their antimicrobial properties and considered the most widely used natural product to date (Nakatsu *et al.*, 2000). The most important antimicrobial application of essential oils are within the fields of dermatology, gastritis, respiratory complaints, wound healing and genital infections (Neuwinger, 2000). Arnald de Villanova, a physician in the 13th century, is believed to be one of the first doctors to have used essential oils therapeutically. By the 17th century essential oils were used extensively by pharmacists where they were applied topically for healing (Hili, 2001). The antiseptic properties of essential oils have been researched since the 1800's, however, during the course of the 19th and 20th centuries the use of essential oils for fragrance and flavour became a more prominent experimental focus rather than the therapeutic application (Burt, 2004). More recently there has been a renewed interest in essential oil research. The resurgence of aromatherapy and aromachology assisted in launching essential oils into the international markets where they are sold as "natural antibiotics". The essential oil of *Melaleuca alternifolia* (tea tree) is probably the best known example, which is sold for its antimicrobial properties. A number of studies have been conducted on the essential oil of *M. alternifolia*. Antiviral activities were investigated where the oil significantly decreased a number of local lesions on *Nicotiana glutinosa* caused by the Tobacco Mosaic virus (Bishop, 1995). Cytotoxicity has also been reported (Söderberg *et al.*, 1996; Hayes *et al.*, 1997; Hammer *et al.*, 2006) but the predominant focus has been on the antimicrobial potential of the oil (Carson *et al.*, 1995; Caelli *et al.*, 2000; Cox *et al.*, 2001;

Christoph and Stahl-Biskup, 2001). Other popular antimicrobial commercial oils include *Lavendula angustifolia* (lavender), *Mentha piperita* (peppermint) and *Rosmarinus officinalis* (rosemary) and *Thymus vulgaris* (thyme). Developing concurrently to this every day use of essential oil products for antimicrobial properties is the scientific exploration which aims at validating the numerous properties ascribed to these natural molecules (Dharmaratne *et al.*, 1999; Iwu *et al.*, 1999). There have been numerous studies on the antimicrobial activities of essential oils in specific plant species. A compendium for the Journal of Essential Oil Research (Lawrence, 1989-2005) documents over ninety antimicrobial publications. These papers include studies on bacteriostatic activities (Clark, 1996; Christoph and Stahl-Biskup, 2001), antifungal activities (Griffin *et al.*, 2000; Dawson-Andoh *et al.*, 2000) and antiviral activities (Bishop, 1995; Chao and Young, 2000). Methodology has been studied extensively due to the problems associated with the volatility and immiscibility of the oil. Diffusion and minimum inhibitory assays (MIC) have pre-dominated many publications and to a lesser extent time-kill methodology (Remmal and Tanaoui-Elaraki, 1993; Hood *et al.*, 2003). The antimicrobial activity of essential oil components has been studied extensively (Hinou *et al.,* 1989; Knobloch *et al.*, 1989; Pauli and Kubeczka, 1997; Pattnaik *et al.*, 1997; Chalchat and Garry, 1997; Nakatsu *et al.*, 2000; Pauli, 2001). Detailed compositional analysis is achieved by gas chromatography and mass spectrometry where the essential oil may yield any number of major constituents. The essential oil constituents that show the highest antimicrobial activity are the phenols followed by the aldehydes, ketones, alcohols, esters and finally the hydrocarbons (Kalemba and Kunicka, 2003). A recent publication (van Zyl *et al.,* 2006) demonstrated the biological activities for 20 essential oil constituents from the seven different structural groups i.e. phenols, aldehydes, ketones, alcohols, esters, terpene hydrocarbons and oxides. Highest antimicrobial activities were found for carvacrol from the phenol group, confirming results from previous studies (Kalemba and Kunicka, 2003). While the study of the antimicrobial properties of essential oil constituents play an important role in determining the overall activity of the plant, it must be taken into account that any of the constituents (which may be more than 70 in any given plant studied) may be responsible for activity. These may be either major or minor constituents or even a combination of constituents that interact synergistically to enhance activity of the plant as a whole. Some *in vitro* antimicrobial combination studies have been undertaken which support the role of synergism in essential oils, but antimicrobial studies have not been undertaken in great depth (Kang *et al.*, 1992; Lachowicz *et al.*, 1998; Cassella *et al.*, 2002).

The objective to include plant-derived antimicrobials in ingestible products to meet the needs of the so called “green consumer” desired by many Westerners has also become very popular (Burt, 2004). Essential oils have become multifunctional i.e. in the reduction and / or elimination of food-borne pathogens, as a preservative and as flavouring agents in food. The use of spices for antimicrobial prophylaxis has been well documented. In a review on the antimicrobial properties of essential oils in food, Burt (2004), listed 36 studies reporting the efficacious use of oils as preservative in various food stuffs. Essential oils, because of their organoleptic nature and flavouring properties meet the culinary need and with their potential medicinal role may play an active role in present and future food preservation. Such applications could contribute significantly as synergistic agents that not only enhance food flavouring but also work prophylactically to ward off undesirable microbial populations.

Due to the known antimicrobial properties of essential oils, an interest has developed in aromatic plants in general, in the continued search for new natural molecules to be used in phytopharmaceuticals (Rates 2001, Cowan 1999).

1.6 The South African perspective

Southern Africa boasts a unique and diverse botanical heritage with over 30 000 plant species of which *ca*. 3 000 species are used therapeutically (van Wyk *et al.*, 1997). Not only is the South African flora rich in diversity but it is also mostly endemic (Mulholland, 2005). In addition to this unique botanical heritage, South Africa has a cultural diversity with traditional healing being integral to each ethnic group. African traditional medicine is the oldest medicinal system, hence culturally often referred to as the Cradle of Mankind (Gurib-Fakim, 2006). The informal verbal record of medicinal use of plant material by the Khoi-San, Nguni, Zulu and the Sotho-speaking ethnic groups has been passed on from generation to generation (Hutchings and van Staden, 1994; van Wyk *et al.*, 1997). There are over 200 000 traditional healers in South Africa who systematically diagnose the cause of disease and thereafter administer plant medicines to holistically improve the health of the patient (van Wyk *et al.*, 1997). Methods such as this are not very different to the western medical system. However, the added benefit of the traditional healer to seek the origin of the ailment makes the use of cultural healing more favourable for many people. There is also the added benefit of availability at low cost, as many “muthi” medicines are sold informally. It has been estimated that over 20 000 tons of plant material are sold annually on the informal medicinal market (Taylor *et al.*, 2001).

Even though conventional drug therapies are the first choice of treatment in first world countries, herbal therapy is a way of life for over 80% of people living in rural South Africa (Shale *et al*., 1999). Indigenous medicinal plant use is well recorded in the readily available local ethnobotanical literature. Despite the well-documented ethnobotanical literature, very little scientific information (e.g. efficacy, phytochemistry) has become available on indigenous medicinally used plants. It is only recently (1999-2006) that a number of findings have emerged on the chemistry and biological activity of plants used in traditional healing. South African contributions within The Journal Ethnopharmacology for the period 1980-1994 were between 10-20%. However, this increased to approximately 55% in the last five years (Light *et al*., 2005). This recent emergence in the scientific validation of South African medicinal plants can possibly be attributed to the number of citations in local books confirming the need for such studies (Hutchings and van Staden, 1994; van Wyk *et al.*, 1997). The National Research Foundation has recently (2005) committed 15 million rand to the Indigenous Knowledge Focus area emphasizing the need to make pharmacognosy-related studies a high priority (Mulholland, 2005).

More specifically, the antimicrobial properties of South African plants have been sorely neglected. The literature search engines "Science Direct" and "Scopus" identified only 4% and 3% (respectively) of citations related to "plants" and "antimicrobial" were of South African origin. While South African researchers seem to be lagging behind on a global scale, the antimicrobial investigation of plants compare favourably with researchers from the rest of Africa (Light *et al*., 2005).

An antimicrobial review of the South African literature reveals a broad spectrum of research activity. Van Wyk (2002), includes a number antimicrobial directives in his review on the ethnobotanical research in southern Africa including social relevance, wound healing, and antidiarrhoeal activity. In addition, there have been a number of antimicrobial screening publications (Rabe and van Staden, 1997; Lin *et al.,* 1999; McGaw *et al*., 2000; Kelmanson *et al*., 2000; Motsei *et al*., 2003; Eldeen *et al*., 2005; Buwa and van Staden, 2006; McGaw and Eloff, 2005). Antimicrobial reports have been undertaken on specific species such as *Helichrysum* (Meyer and Afolayan, 1995; Meyer and Dilika, 1996; Meyer *et al*., 1997; Dilika *et al*., 1997; Afolayan and Meyer, 1997; Mathekga and Meyer, 1998; Lourens *et al*., 2004; van Vuuren *et al*., 2006) where research groups have focused their expertise on specific plant groups. There has also been local research activity on medicinal plants used to treat sexually transmitted

infections (Tshikalange *et al*., 2005) and mycobacterial studies (Lall and Meyer, 1999). Other research groups have focused their studies on the microbiological activity of particular botanical families e.g. the Combretaceae (Eloff, 1999; Martini *et al*., 2004; Eloff *et al*., 2005). Furthermore, there have been significant contributions toward the methods associated with biological testing of botanical material. Of the numerous methodology publications undertaken, the most prominent has been the minimum inhibitory concentration determination publication by Eloff, 1998a which has been extensively cited.

Other microbial related botanical studies have involved the isolation of antimicrobial compounds from South African plant species. This has been successfully undertaken where the need for a multi-disciplinary approach is integral to achieve high quality research. Collaboration between plant chemists, botanists and microbiologists have yielded some valuable contributions (Rabe and van Staden, 2000; Drewes *et al*., 2005; Drewes *et al*., 2006). Other studies focusing on specific parts of the plant such as roots, stems or leaf material have also been investigated (Louw *et al*., 2002; Lewu *et al*., 2006).

Even though South African researchers have made valuable contributions towards ethnopharmacological investigations, the need exists to capitalize on the rich botanical diversity and meet the global need for the compilation of significant monographs. This is necessary in order to meet The World Health Organization's objective to provide guidelines for the assessment of herbal medicines.

The examination of the antimicrobial properties of aromatic plants endemic to South Africa and used in traditional healing forms an integral focus of this thesis. One of the main objectives is to make a significant contribution towards generating microbiological data to validate the traditional use and thus ultimately contribute towards the acceptance of plant-based antimicrobials in westernized healthcare systems.

1.7 Thesis structure

The thesis is introduced by a general introduction followed by individualised studies on nine different plant species. In order to avoid methodology repetition reference is made to preceding Chapters. A flow chart (Figure 1.3) presents how each study was systematically followed by initial plant collection and identification. Thereafter chemical analysis of the essential oil for

each specific species was undertaken. Antimicrobial screening and viability monitoring followed with further in-depth target assay applications. Findings from each investigation have been presented at a conference, published or have been submitted for review. Abstracts of publications are given in the Appendix. A selection of indigenous oils is comparatively evaluated with popular commercial oils to correlate antimicrobial efficacies. A general methodology Chapter follows which encompasses an overview of all methods followed as well as trouble shooting, literature sourced and many of the challenges encountered when studying the various different plant species. A final conclusion is provided to integrate all Chapters and provide some recommendations and guidelines for further ethnopharmacological antimicrobial studies.

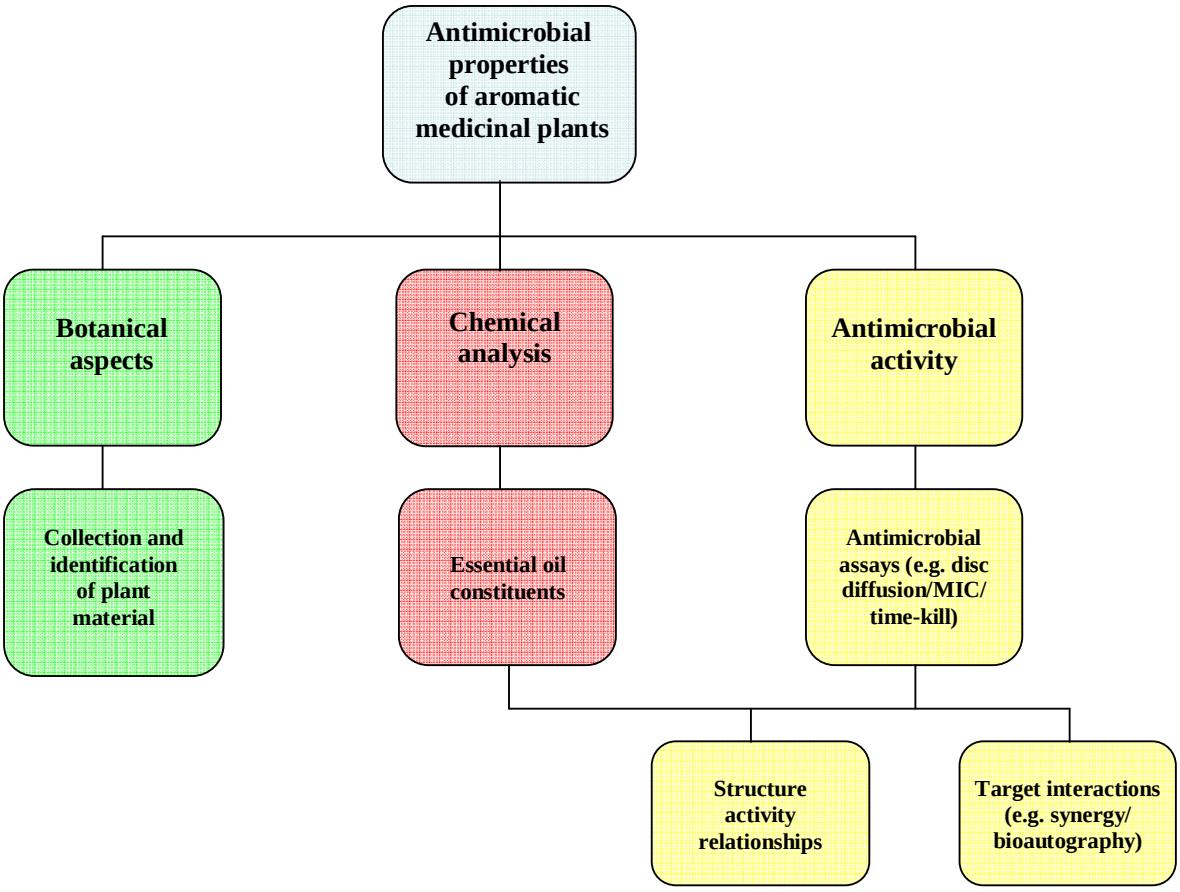


Figure 1.3 A schematic outlay of the study protocol for the antimicrobial investigation of aromatic plants.

1.7.1 Study objectives

The overall intention of this study is to provide a scientific basis for the use of selected traditional aromatic plants used therapeutically for conditions associated with infectious diseases.

Within this framework, the following objectives were proposed;

1. To determine the antimicrobial activity of selected indigenous aromatic plants used in traditional healing.
2. To record the essential oil composition of some of the most widely used South African medicinal aromatic plants.
3. To investigate the plant composition with antimicrobial activity relating to structure activity relationships and if possible identify the antimicrobial factor responsible.
4. To study specific pharmacological interactions.
5. To compare the antimicrobial activity (potency) of indigenous aromatic plants to commercially available essential oils with claimed antimicrobial activity.
6. To compare and validate results obtained from various test methodologies (e.g. disc diffusion, MIC and time-kill studies).
7. To provide a scientific rationale for the traditional use of various medicinal aromatic plants to treat infectious diseases.

Chapter 2

Myrothamnus flabellifolius Welw., antimicrobial efficacy determined by disc diffusion, minimum inhibitory concentration and time-kill methodology.

2.1 Introduction

Myrothamnus flabellifolius Welw. (Myrothamnaceae) is a plant used traditionally by many of the ethnic people of Africa. It has been used medicinally by the Pedi, Zulu, Shona healers and early Rhodesian settlers (Watt and Breyer-Brandwijk, 1962). The use of leaves and stems as flavouring agents has also been reported (van Wyk and Gericke, 2000). Furthermore, the use of *M. flabellifolius* to treat psychological tribulations is strongly linked to the unusual nature of the plant to revive to its original vegetative state even when completely desiccated. In the rainy season the seemingly dead plants are simply revived from the dormant state. This remarkable feature of the plant is used by the traditional healer to explain the healing action of the plant. The patients are told to take the dry plant home with them, being a symbol of both their psychological and physical state. They are told to place the dried plant in water and as the plant revives to it's original state, so too will the patient become well again. This property is reflected in the English (resurrection bush) and Zulu (uvukwabafile) vernacular names and is used as a symbol of hope in traditional African treatment against severe depression (Credo Mutwa, personal communication to Prof. B-E van Wyk).

Due to the conflicting reports on the composition of the essential oils distilled from *Myrothamnus flabellifolius*, the oil composition has been re-examined. Some of the major compounds previously identified in the essential oils are pinocarvone and *trans*-pinocarvone (Chagonda *et al.*, 1999) and camphor, α-pinene and 1,8-cineole (van Wyk *et al.*, 1997). The antimicrobial properties are investigated here using disc diffusion, minimum inhibitory concentrations (MIC) and time-kill methods.

2.2 Botanical description

Myrothamnus flabellifolius is a small woody shrub about 0.4 m in height. The small aromatic leaves (Figure 2.1) are fan-shaped and conspicuously toothed on the upper leaf margin. Plants are monoecious bearing inconspicuous flowers. The leaves have a strong aromatic character. The plants have an affinity for rocky areas and are usually wedged into crevices of large boulders as illustrated in Figure 2.2 (van Wyk *et al.*, 1997).



Figure 2.1 Aromatic foliage of *Myrothamnus flabellifolius.*



Figure 2.2 *Myrothamnus flabellifolius* in habitat.

2.3 Distribution

Myrothamnus flabellifolius grows on rocky outcrops in the northern regions of South Africa from Gauteng, extending through Mpumalanga to the Northern Province and Botswana. It is also found in central and northern Namibia (Figure 2.3).

2.4 Medicinal uses

The leaves and twigs are used in many medicinal preparations. The main traditional uses related to microbial infections as summarized from van Wyk *et al.* (1997) and Hutchings *et al.* (1996) include: infusions taken for colds, respiratory ailments and decoctions taken orally to alleviate kidney problems. Externally, the plant may be used to treat abrasions and the dried powdered

leaves are used in dressings for burns and wounds, possibly to prevent sepsis. Lotions incorporating *M. flabellifolius* are prepared and applied to skin abrasions (Watt and Breyer-Brandwijk, 1962). Leaves may be burnt and the smoke inhaled to treat chest pains and asthma. The smoke may also be directed into the vagina to treat infections and pains in the uterus (van Wyk and Gericke, 2000). The local Pedi people of northern South Africa smoke the leaves in pipes to alleviate chest pains. The Karanga communities chew the aromatic leaves for mouth complaints. In central Africa the plant is used as a tonic and as a treatment for breast complaints. Shona healers have used the plant to treat epilepsy, madness and coughs.

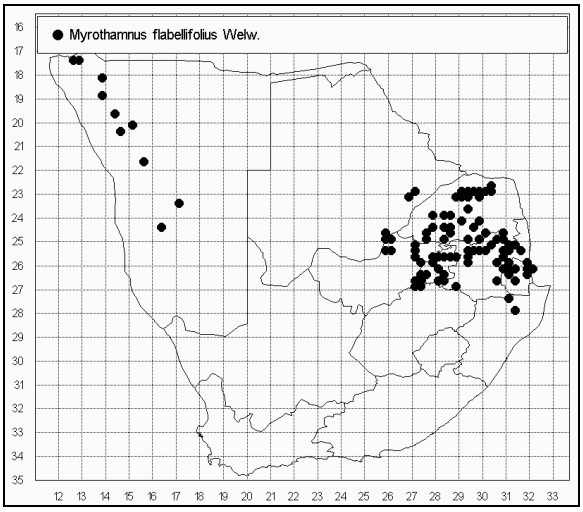


Figure 2.3 The geographical distribution of *M. flabellifolius* in South Africa (SANBI).

2.5 Methods

2.5.1 Chemical aspects

Plant collection: The aerial parts were collected in the growing season (March) from a population growing on rocky slopes in Klipriviersberg, Johannesburg, South Africa. A voucher specimen (Table 2.1) is retained in the Department of Pharmacy and Pharmacology, University of Witwatersrand. Due to the large volume of oil required for time-kill studies plant material was collected from several plants in the same population.

Table 2.1 Plant collection data for *M. flabellifolius.*

Plant	Voucher	Material distilled (g)	Essential oil yield (% w/w)
M. flabellifolius	SVV934	2700.7	0.7

Distillation of essential oils: Distillation protocols were undertaken according to Guenther (1948). Fresh plant material was hydrodistilled in a Clevenger type apparatus (Figure 2.4) and the essential oil collected after three hours. A round bottom flask of five litre capacity is packed with fresh leafy plant material. Approximately 500 mL distilled water is added and heated by means of an electronic heating mantel. The essential oils were then condensed in a cooling system where they were collected. The temperature was kept constant by letting the water run through the condenser. Thereafter, the oil was allowed to stand at room temperature to allow for good separation and collected in amber vials for storage at 4 °C.



Figure 2.4 Clevenger apparatus used for distillation of plant material.

Gas chromatography (GC): Due to conflicting reports of the major constituents within *M. flabellifolius* (Da Cunha, 1974; Gibbs, 1974; Chagonda *et al*., 1999) and the large amounts of oil required for the antimicrobial testing the oil obtained from each of the plants were analyzed by GC to determine any chemical variation. Analysis was performed on a Shimadzu 17A gas chromatograph using the following parameters; Column: J&W-DB1 (60 m x 0.25 mm id., 0.25 µm film thickness); Temperatures: injection port 230 °C, column 60 °C for 1 min, 5 °C / min to 180 °C, 180 °C for 2 min, (total = 25 min). A small amount (1 µl) of oil sample was injected with 0.85 µl hexane (Rochelle) into a stream of an inert gas (helium), where the separated components then emerge from the column at discrete intervals (characteristics for each component) and pass through a flame ionization detector (FID). Chromatograms were recorded from the time of initial elution of compounds. The retention times were used for qualitative identification with peak r areas to provide qualitative information.

Gas chromatography combined with mass spectrometry (GC-MS): Oil samples (0.1 µl in 0.9 µl hexane) were further quantitatively analyzed with GC-MS using the Hewlett-Packard 1800A GCD system operating under the following conditions: column; HP-Innowax (60 m x 0.25 mm id., 0.25 µm film thickness) was used with helium as a carrier gas; Temperatures: injection port 250 °C, column 60 °C for 10 min, 4 °C / min to 220 °C, 220 °C for 10 min, 1 °C / min to 240 °C (total = 80 min). The split ratio was adjusted to 50:1. The MS were taken at 70 eV. The mass range was from *m/z* 35 to 425. Data was recorded after a 5 min lag phase. Relative percentages of the separated compounds were calculated from total ion chromatogram by the computerized integrator. The MS were compared with those of reference compounds and confirmed with the aid of retention indices from published sources using the in-house Başer Library of Essential Oil Constituents in collaboration with Prof Başer at the Department of Pharmacognosy, Faculty of Pharmacy, Anadolu University, Eskişehir, Turkey.

2.5.2 Antimicrobial aspects

Culture and media preparation: The NCCLS (2003) guidelines were used to ensure that accurate microbiological assay and transfer techniques were followed. All stock cultures were obtained from the National Health Laboratory Services (NHLS) with the exception of *Candida albicans*, which was obtained from the South African Bureau of Standards (SABS). Authorization to keep and use cultures for research purposes was obtained from the Department of Health (reference: J1/2/4/16 NO1). Table 2.2 lists the cultures with corresponding reference number (where possible) used in this study. Stock cultures were retained at –20 °C, subcultured onto Tryptone Soya (Oxoid) agar, incubated at optimum temperatures and checked for purity. Isolated pure colonies were selected and transferred onto Tryptone Soya (Oxoid) agar and thereafter kept viable by subculturing weekly for stock culture maintenance. All media was prepared according to the instructions provided by the supplier i.e. weighed, dissolved in distilled water and autoclaved (Butterworth) at 121 °C for 15 min. After sterilization, all media was pre-incubated to confirm sterility before further use.

Disc diffusion assays: Disc diffusion assays as described by Janssen *et al.* (1987), Pauli and Kubeczka (1997) and Hewitt and Vincent (2003) and were performed on the hydrodistilled oils. Base layers of Mueller Hinton (Oxoid) agar were prepared for bacterial studies and depending on the size of the assay plate either 15 mL or 100 mL of pre-sterilized molten agar was poured into a sterile petri dish. Care was taken so that the agar was maintained at a working temperature of

50-55 °C. A temperature higher than 55 °C may result in either killing or reducing the viable count of the test organism. Temperatures lower than 50 °C will result in the agar rapidly solidifying making uniformity of the agar surface problematic. Similarly, disc diffusion assays for fungal strains were prepared with the exception that Sabouraud's Dextrose (Oxoid) agar was used. Spore suspensions yielding an approximate inoculum size of 1 x 10^8 CFU/mL were thoroughly mixed into the overlaying agar surface of identical volume. With aseptic manipulation, 6 mm discs were saturated with the pure oil (approximately 9 mg) and were placed onto the set agar. Neomycin discs (30 µg, Oxoid) were used for bacterial controls and Nystatin discs (100 IU, Oxoid) were used for fungal controls. These positive controls were included in all test replicates and repetitions to ensure susceptibility of micro-organisms to conventional antimicrobials. Negative controls i.e. sterile discs were used to ensure no pre-existing antimicrobial activity was evident. All plates were allowed to pre-diffuse at 4 °C for one hour to allow for the dispersion of essential oil from the disc into the set agar before incubation at 37 °C for 24 hr, with the exception of the two yeasts *Candida albicans* and *Cryptococcus neoformans* which were incubated at 37 °C for 48 hr and the moulds *Aspergillus niger* and *A. alternata* which were incubated at 25 °C for seven days. Results were recorded at least in triplicate and the mean of the zone of inhibition measured from disc edge to margin of culture growth is documented in Table 2.4.

Minimum inhibitory concentrations (MIC): After preliminary screening by disc diffusion assay**,** the broth MIC micro-dilution bioassay was determined (Carson *et al.*, 1995; Eloff, 1998a; NCCLS, 2003). All bacterial cultures were subcultured from stock agar plates and grown in Tryptone Soya (Oxoid) broth overnight. Yeasts were incubated for a further 24 hr. Microtitre plates were aseptically prepared by the addition of 100 µl distilled, sterile water into each well. The hydrodistilled *M. flabellifolius* oils at starting stock concentrations of 128 mg/mL were subsequently transferred into the first rows of a microtitre plate. Essential oils were made up to concentration with acetone (Merck) as the solvent of choice. Negative controls (acetone in microtitre plate without antimicrobial) were included in each assay. Serial dilutions are performed on the *M. flabellifolius* oils so that concentrations of 32, 16, 8, 4, 2, 1, 0.5 and 0.25 mg/mL were obtained. The overnight cultures were diluted in fresh Tryptone Soya broth at a 1:100 ratio, yielding an approximate inoculum size of 1 x 10^8 colony forming units (CFU)/mL and 100 µl was added to all wells. The microtitre plates were sealed with sterile adhesive sealing film (AEC Amersham) to ensure that no loss of essential oil took place. Optimal (37 °C for 24 hr for bacteria and 48 hr for yeasts) incubation conditions followed.

Table 2.2 Microbial organisms with corresponding reference numbers and acceptable MIC ranges for controls (ciprofloxacin for bacteria and amphotericin B for yeasts).

Test organism	MIC controls (μg/mL)
Pseudomonas aeruginosa (ATCC 9027)	0.25-2.50[*2]
***Escherichia coli* (ATCC 25922)**	**0.004-0.016[*1]**
Salmonella typhimurium (ATCC 14028)	0.63-12.50[*2]
Proteus vulgaris (clinical strain)	0.25-128[*2]
Serratia odorifera (ATCC 33132)	0.30-1.25[*2]
***Staphylococcus aureus* (ATCC 25923)**	**0.12-0.50[*1]**
***Enterococcus faecalis* (ATCC 29212)**	**0.25-2.00[*1]**
Cryptococcus neoformans (ATCC 90112)	0.625-2.500[*2]
Candida albicans (ATCC 10231)	1.25-2.50[*2]
Aspergillus niger (clinical strain)	ND[*3]
Alternaria alternata (clinical strain)	ND [*3]

[*1] NCCLS, 2003; Andrews, 2004.
[*2] Acceptable ranges as determined within the Department of Pharmacy and Pharmacology, University of Witwatersrand (controls in this study were run for all test organisms even though only one organism from that indicated in bold is recommended for inclusion in each MIC assay).
[*3] No MIC's were performed on moulds due to the extended incubation requirements.

Positive bactericidal controls i.e. ciprofloxacin (Sigma-Aldrich) at starting stock concentrations of 0.01 mg/mL were included in each assay to confirm the antimicrobial susceptibility. The ciprofloxacin stock solution was prepared aseptically with sterile water. The amphotericin B (Sigma-Aldrich) stock solution for yeast controls was initially diluted with dimethyl sulfoxide (DMSO, Merck) as recommended by the supplier and thereafter diluted with sterile water to a starting concentration of 0.01 mg/mL. The selection of ciprofloxacin and amphotericin B as suitable reference controls is in support of the NCCLS guidelines (2003). A review article (Lahlou, 2004), on the methods in assessing the bio-activity of essential oils also cites a number of publications in support of ciprofloxacin and amphotericin B as standard controls. Conventional antibiotics inhibit microbial growth at certain acceptable quantitative MIC ranges. Any variations to the acceptable MIC values indicate either strain resistance (if the MIC value is higher than that from the range) or unusual strain susceptibility (if the MIC value is lower than the range). This would impact on the "true" results obtained for the test and therefore monitoring these ranges is important to corroborate the accuracy of results if further clinical studies are to be

undertaken. Documented control ranges are only available for selected pathogens, however, all assays were run with micro-organism controls of the particular pathogen studied. If control guidelines were unavailable, at least six repetitions and replications on controls were done to achieve a standard guideline from which comparison could be made. Table 2.2 gives the acceptable ranges for ciprofloxacin and amphotericin B against pathogens used in the MIC assays. A 0.4 mg/mL *p*-iodonitrotetrazolium violet (Sigma-Aldrich) solution (INT) was prepared and 40 µl transferred to all inoculated wells. The use of INT to determine the end point MIC value is based on the principle of detection of dehydrogenase activity where the metabolically active test organism reduces INT to a red-purple colour. The microtitre plates inoculated with bacteria were examined after six hours to determine a colour change in relation to concentration of microbial growth. The yeasts were examined after 24 hr. The lowest concentration having no colour change is defined as the MIC. Minimum inhibitory assays were done in triplicate and the mean tabulated (Table 2.4).

Time-kill methodology: Death kinetic assays, as described by Christoph and Stahl-Biskup (2001) were performed on a Gram-positive organism *S. aureus* (ATCC 25923), a Gram-negative organism *P. aeruginosa* (ATCC 9027) and a yeast *C. albicans* (ATCC 10231). Stock cultures were grown in Tryptone Soya (Oxoid) broth and centrifuged (MSE) for 10 min at 5000 rpm. The supernatant was discarded and the pellets resuspended in a 0.9% NaCl (Labchem) solution. Oil concentrations of 0.125, 0.5, 0.75, 1 and 2% (1.8, 5.2, 8.1, 9.5 and 18.1 mg/mL respectively) were incorporated into 50 mL Tryptone Soya broth with 0.5% Tween 80 (Saarchem) and a final inoculum of approximately 1 x 10^8 CFU/mL. The different concentrations were incubated at 37 °C with agitation in a shaking water bath (Labotec). At pre-determined time intervals ranging from 0 min - 24 hr, (0, 5, 15, 30, 60, 120, 240 min and 24 hr), aliquots of 1 mL were transferred to 9 mL inactivation broth consisting of 0.1% peptone (Oxoid), 5% lecithin (Merck) and 5% yeast extract (Oxoid). A further four serial dilutions were performed from the inactivation broth into 9 mL of 0.9% NaCl. From each dilution 100 µl was plated onto Tryptone Soya (Oxoid) agar. The plates were incubated (37 °C for 24 hr for bacteria and 48 hr for the yeast) and colony-forming units (CFU/mL) counted. Death kinetics were expressed in $\log_{10}$ reduction time-kill plots.

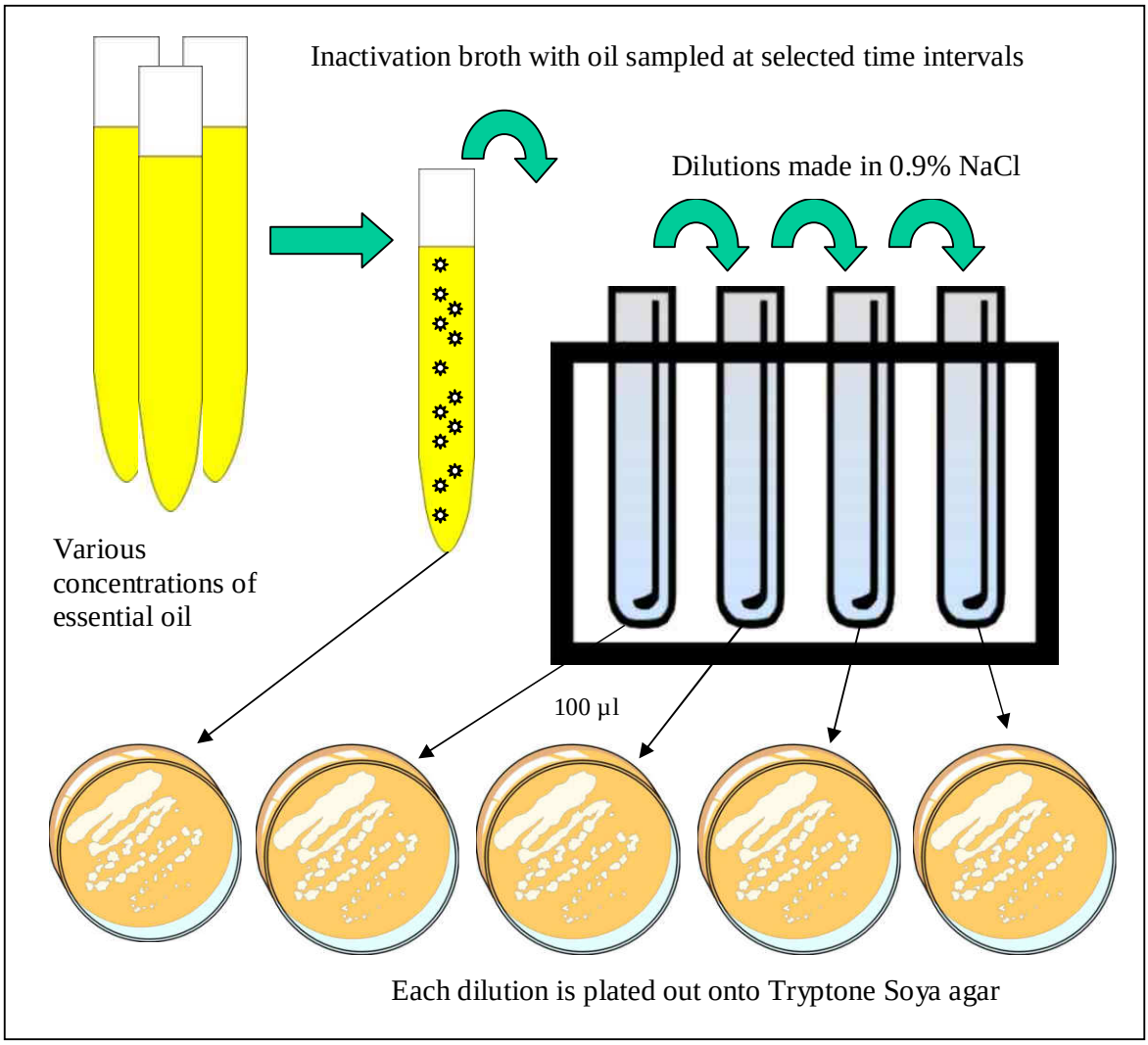


Figure 2.5 A diagrammatic representation of the steps undertaken for each oil concentration carried out at all time intervals.

Negative controls were included in all time-kill assays having the same broth formulation, inoculum and Tween 80 but without the oil. This control validated the assay conditions so that the test organism's growth could be monitored when no inhibitor was added. Positive controls, ciprofloxacin for bacterial assays, or amphotericin B for fungal assays at a concentration of 0.063% (0.6 mg/mL) were included in all time-kill assays. This positive control was included to ensure microbial death response to conventional antimicrobials. Each time-kill plot represents the mean of values where the kinetics were determined in duplicate and each figure represents the time period at which most activity could be noted. Various steps in the method are seen in Figure 2.5 and 2.6.



2.6 Results and discussion

2.6.1 Essential oil chemistry

The GC analysis of individual plants within the Klipriviersberg locality indicated consistent chemical composition (data not shown) and consequently the oil obtained from individual plants were pooled and analyzed with GC-MS. Eighty-four compounds were identified in the hydrodistilled *M. flabellifolius* essential oil representing 86% of the total composition (Table 2.3).

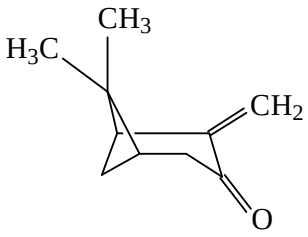
Table 2.3 The essential oil composition and integration percentage of *M. flabellifolius* as determined by GC-MS.

RRI*	Compound name	%
1032	α-pinene	1.6
1076	camphene	0.1
1118	ß-pinene	0.2
1132	sabinene	tr
1174	myrcene	0.2
1203	limonene	6.1
1213	1,8-cineole	0.2
1218	ß-phellandrene	tr
1220	*cis*-anhydrolinalool oxide	tr
1224	*o*-mentha-1(17)5,8-triene	0.2
1280	*p*-cymene	0.2
1384	α-pinene oxide	tr
1408	1,3,8-*p*-menthtriene	0.1
1435	γ-campholene aldehyde	tr
1452	α-*p*-dimethylstyrene	0.2
1466	α-cubebene	tr
1468	*trans*-1,2-limonene epoxide	tr
1482	fenchyl acetate	0.1
1497	α-copaene	0.8
1528	α-bourbonene	tr
1535	ß-bourbonene	0.2
1536	pinocamphone	0.1
1541	benzaldehyde	tr
1549	ß-cubebene	0.1
1553	linalool	1.8
1562	isopinocamphone	0.2
1565	linalyl acetate	0.2
1571	*trans*-*p*-menth-2-en-1-ol	tr
1586	**pinocarvone**	**11.1**
1597	bornyl acetate	1.8
1600	ß-elemene	0.5
1611	terpinen-4-ol	0.4
1616	hotrienol	0.3
1624	*cis*-dihydrocarvone	0.1
1639	*trans*-*p*-mentha-2,8-diene-1-ol	2.7
1648	myrtenal	0.9
1658	sabinyl acetate	0.1
1661	alloaromadendrene	0.1
1661	*trans*-pinocarvyl acetate	tr
1664	***trans*-pinocarveol**	**19.6**
1678	*cis*-*p*-menth-2,8-dien-1-ol	2.3
1687	α-humulene	0.1
1697	carvotanacetate	tr
1704	γ-muurolene	tr

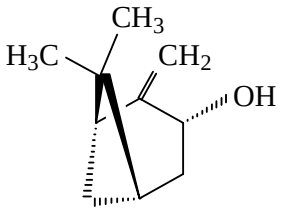
RRI*	Compound name	%
1706	α-terpineol	0.3
1719	borneol	tr
1726	germacrene D	1.1
1740	α-muurolene	0.7
1747	*trans*-carvyl acetate	0.4
1751	carvone	1.1
1758	*cis*-piperitol	3.7
1773	δ-cadinene	0.6
1776	γ-cadinene	0.2
1797	*p*-menthyl acetophenone	0.1
1802	cuminaldehyde	0.1
1804	myrtenol	1.1
1807	perilla aldehyde	0.1
1811	*trans-p*-menth-1-(7)-8-diene-2-ol	7.4
1845	*trans*-carveol	1.3
1853	*cis*-calamenene	0.1
1865	isopiperitenone	0.3
1882	*cis*-carveol	0.2
1896	*cis-p*-menth-1-(7)-8-diene-2-ol	7.0
1941	α-calacorene I	0.1
1945	1,5-epoxy-salvial-4(14)-ene	tr
1953	palustrol	0.1
1956	*p*-isopropyl benzaldehyde	0.1
1984	α-calacorene II	0.1
2008	caryophyllene oxide	0.1
2037	salvial-4(14)-en-1-one	0.1
2057	ledol	0.3
2069	germacrene D-4ß-ol	0.1
2080	cubenol	0.3
2088	1-epi-cubenol	0.4
2104	viridiflorol	0.2
2113	cumin alcohol	0.1
2144	spathulenol	0.8
2187	T-cadinol	0.9
2209	T-muurolol	1.5
2219	δ-cadinol	0.6
2255	α-cadinol	3.0
2264	intermedeol	0.2
2273	selin-11-en-4-αol	0.4
2324	caryophylladienol II (=*caryophylla-2(12),6(13)-dien-5α-ol*)	0.1
	TOTAL	**86.00**

*RRI: Relative retention indices as eluted from a polar column.
% calculated from TIC data
tr = trace <0.1%.

Major compounds: The two major compounds (Figure 2.7) in the essential oil of *M. flabellifolius* are *trans*-pinocarveol (19.6%) and pinocarvone (11.1%), representing 30.7% accumulatively.



pinocarvone



trans-pinocarveol

Figure 2.7 Chemical structures for major compounds identified in the essential oil of *M. flabellifolius.*

The major compounds in the essential oil of *M. flabellifolius* have previously been reported by Da Cunha (1974), indicating carvone and perillic acid as the major constituents. Gibbs (1974) recorded the presence of 1,8-cineole and diosphenol in the essential oil. Chagonda *et al.* (1999), identified 43 compounds from *M. flabellifolius* with *trans*-pinocarveol (28.7-28.8%), pinocarvone (13.4-21.3%), α-pinene (tr-23.0%) and β-selinene (5.0-9.9%) as major constituents. The major constituents (Figure 2.7) detected in this study could not corroborate with the results reported by Da Cunha (1974) and Gibbs (1974). The composition however, corresponds to the analysis reported by Chagonda *et al.* (1999) who also recorded *trans*-pinocarveol and pinocarvone as major constituents. Although no variation was recorded within the Klipriviersberg area, more populations representing the wide geographical distribution range of this plant may yield information on possible chemotypic variations.

2.6.2 Antimicrobial activity

Myrothamnus flabellifolius have been used traditionally to treat various ailments, some associated with microbial infections (Neuwinger, 2000). The method of administration, for example the inhalation of smoke from the burnt leaves indicates the probability that essential oils are responsible for the biological activity. An investigation of the antibacterial and antifungal activity of *M. flabellifolius* against eleven different pathogens by disc diffusion assay (Table 2.4)

showed inhibition for all micro-organisms except *S. typhimurium* and *A. alternata*. While results for bacterial isolates show minimal (1 mm for *P. aeruginosa*) to moderate sensitivity (3 mm for *S. aureus, E. coli* and *S. odorifera*), it was interesting to note that the fungal isolates *C. neoformans* and *A. niger* showed marked antimicrobial activity (8 mm and 10 mm respectively) in the essential oil of *M. flabellifolius.* These two fungal organisms are respiratory pathogens and may enter the host via the respiratory tract. Cryptococcosis is a chronic infection involving mainly the lungs and meninges and *A. niger* is implicated in bronchopulmonary lung infections (Boyd and Hoerl, 1981). As the smoke from *M. flabellifolius* is inhaled directly into the lungs, it provides some rational into its possible antimicrobial use in traditional medicine. The notable candidacidal activity reported here (5 mm) could possibly provide scientific support for the traditional use of *M. flabellifolius* for *Candida*-related infections (e.g. mouth and vaginal infections).

Table 2.4 Zones of inhibition (measured in mm from disc edge to margin of culture growth) and MIC (mg/mL) of *M. flabellifolius* essential oil.

Test organism	Disc diffusion (mm)	Disc diffusion Control*[1]	MIC (mg/mL)	MIC controls*[2]
Staphylococcus aureus (ATCC 25923)	3	7	4	0.30×10^{-3}
Enterococcus faecalis (ATCC 29212)	2	4	8	2.00×10^{-3}
Pseudomonas aeruginosa (ATCC 9027)	1	1	8	0.80×10^{-3}
Escherichia coli (ATCC 25922)	3	5	4	0.02×10^{-3}
Proteus vulgaris (clinical strain)	2	6	4	1.60×10^{-4}
Serratia odorifera (ATCC 33132)	3	6	4	0.30×10^{-3}
Salmonella typhimurium (ATCC 14028)	0	4	4	0.80×10^{-3}
Candida albicans (ATCC 10231)	5	7	4	1.30×10^{-3}
Cryptococcus neoformans (ATCC 90112)	8	10	2	2.50×10^{-3}
Aspergillus niger (clinical strain)	10	10	ND*[3]	ND*[3]
Alternaria alternata (clinical strain)	0	2	ND*[3]	ND*[3]

*[1] Neomycin and nystatin served as controls for bacteria and fungi respectively.
*[2] Ciprofloxacin and amphotericin B served as controls for bacteria and yeasts respectively.
*[3] ND = Methods did not incorporate MIC's for moulds.

In addition to disc diffusion assays, quantitative MIC assays was also undertaken on the same bacterial and yeast strains (Table 2.4). Congruency was mostly obtained between the two methods. Where disc diffusion assays showed moderate sensitivity (3 mm) similar moderate activities (4 mg/mL) were found in the MIC assay. Similarly, where lower sensitivities were obtained in the disc diffusion assay (1-2 mm), MIC studies indicated less activity (8 mg/mL). Results for *C. neoformans* indicated one of the highest sensitivities in the disc diffusion methodology (8 mm) and highest sensitivity in the MIC assay (2 mg/mL), however, some variation did occur. *Salmonella typhimurium* having no activity in the disc diffusion assay showed moderate sensitivity in the MIC assay (4 mg/mL) and conversely *C. albicans* having high sensitivity (5 mm) in the disc diffusion assay showed only moderate activity (4 mg/mL) in the MIC assay. Such method variations are not unexpected when investigating essential oils as results may be influenced by the choice of growth medium (agar in disc diffusion and broth in MIC), diffusion rate, oil volatility, water insolubility, and the oil complexity (Janssen *et al.*, 1987).

Researchers often find the reproducibility of plant-based antimicrobial studies problematic (Janssen *et al.*, 1987; Pauli and Kubeczka, 1997; Njenga *et al.*, 2005). To further substantiate the results obtained for the antimicrobial disc diffusion and MIC assays, time-kill studies were implemented. As this method involved a number of fine dilution parameters and was undertaken for the first time within the Department, tandem assays were undertaken using similar methods under the guidance of Prof. Klepser at the University of Iowa. This international collaboration to standardize and confirm results obtained from both laboratories were in agreement and further comparative analysis is discussed in Viljoen *et al.* (2002).

Three susceptible organisms were selected to demonstrate the rapid onset of antimicrobial activity using time-kill methodology. The bactericidal activity of *M. flabellifolius* against *S. aureus* was investigated due to its comparatively high antimicrobial activity seen in the disc diffusion assay (Table 2.4). *Staphylococcus aureus* is associated with primary infections of the skin, such as boils, carbuncles and impetigo, thus providing some rationale for the traditional use to treat skin abrasions. Even though limited antimicrobial activity was noted for *P. aeruginosa* in the disc diffusion assay, further time-kill investigation was undertaken based on the hypothesis that *P. aeruginosa,* could possibly be the causative organism responsible for infections from burn wounds. As *M. flabellifolius* is traditionally used as a dressing for burns and wounds, possible

preventative anti-infective properties may play a role in therapeutic treatment. Lung disease and pseudomonial pneumonia also directly correlates with medicinal preparations inhaled by the ethnic Pedi community and warranted further antimicrobial investigation with *P. aeruginosa*. *Candida albicans* was selected for an in-depth fungal time-kill investigation due to its moderate antimicrobial susceptibility, as seen in the MIC assay (4 mg/mL) and susceptible profile noted in the disc diffusion assay (5 mm), but more due to the prevalent pathogenesis. More than 80% of all fungal bloodstream infections are a direct result from the *Candida* species and since the eighties there has been an explosive rise in the rate of Candidal infections (Klepser *et al.*, 1998).

The death kinetics of *S. aureus, P. aeruginosa* and *C. albicans* are represented as time-kill curves ($\log_{10}$ reduction values) in Figures 2.8, 2.9 and 2.10 respectively. Positive controls (ciprofloxacin and amphotericin B) presented with an immediate death response and due to presentation scale were not included in the time-kill plots. Negative controls, having no essential oil (indicated in black in Figures 2.8, 2.9 and 2.10) show a continuous growth pattern. For *S. aureus* (Figure 2.8), the essential oil generally followed a concentration-dependent antibacterial trend. An oil concentration of 0.125% and 0.25% resulted in a decrease in colonies in comparison with the control, however, no cidal action was noted. A bactericidal effect after four hours was observed at 0.75% (8.1 mg/mL) and a more rapid killing rate within one hour for *M. flabellifolius* oil at concentrations 1% (9.5 mg/mL) and 2% (18.1 mg/mL). The bactericidal effect noted here correlates to a degree with the MIC values (4 mg/mL) where minimal growth was still noted in the time-kill assay at 0.5% (5.2 mg/mL).

For *P. aeruginosa* (Figure 2.9), death kinetics indicate a rapid decrease in the number of surviving cells within 60 min at higher concentrations (1% and 2%) but the CFU's increased over time. Lower concentrations (0.125%-0.75%) also presented an initial decrease in CFU but not as prominently observed as in higher concentrations. This suggests that the oil produces an initial bacteriostatic effect but regrowth of the organism occurs with time. This regrowth seen in the time-kill assay gives a more detailed account of the essential oil action on *P. aeruginosa*, not seen in the disc diffusion nor MIC assays where the final limited antimicrobial effect was only observed after 24 hr. Tam *et al.* (2005), highlighted possible reasons for encountering regrowth during time-kill studies. Previous studies (Mouton *et al.*, 1997) have postulated that regrowth may be attributed to the possibility of delayed growth of sub-populations. These organisms growing at a slower rate allow for resistance mechanisms to be transferred to other sub-populations thus enabling the population to withstand exposure of the antimicrobial test

substance. This would explain the initial reduction in colony forming units and later regrowth where microbial populations have been exposed to the essential oil over time. Another postulation explaining regrowth (Tam *et al.*, 2005) is simply by means of adaptation where continuous exposure to the test antimicrobial eradicates sub-populations over time until, the most resistant microbial population becomes increasingly difficult to kill over time.

All the oil concentrations were fungicidal for *C. albicans* (Figure 2.10), with rapid killing rates within the first four hours. The highest oil concentration (2%) was fungicidal within 5 min. The death kinetic comparison between the three organisms studied showed that the killing rate was greater for *C. albicans*, then *S. aureus*, and lastly only initial activity for *P. aeruginosa*. These time-kill studies correlate directly with the disc diffusion and MIC results, which as an initial screening procedure also showed antimicrobial activities highest for *C. albicans* and lowest for *P. aeruginosa* of the three pathogens studied. The value of time-kill methodology over other methods is clear as it demonstrates a more descriptive means of explaining the pharmocodynamic interaction of the oil with test organism over time, not seen with methods such as disc diffusion and MIC where results are observed at the end of an observation period. Even though the investigation is *in vitro*, a more realistic approach is given to what may possibly be occurring in an *in vivo* situation.

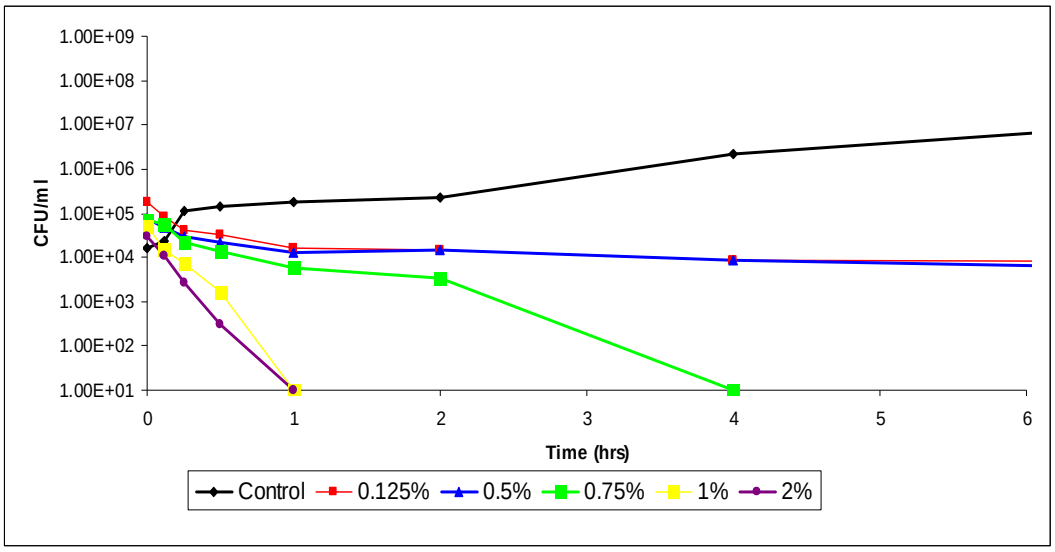


Figure 2.8 Time-kill expressed in Log_{10} reduction of *S. aureus* exposed to *M. flabellifolius* essential oil within the first six hours.

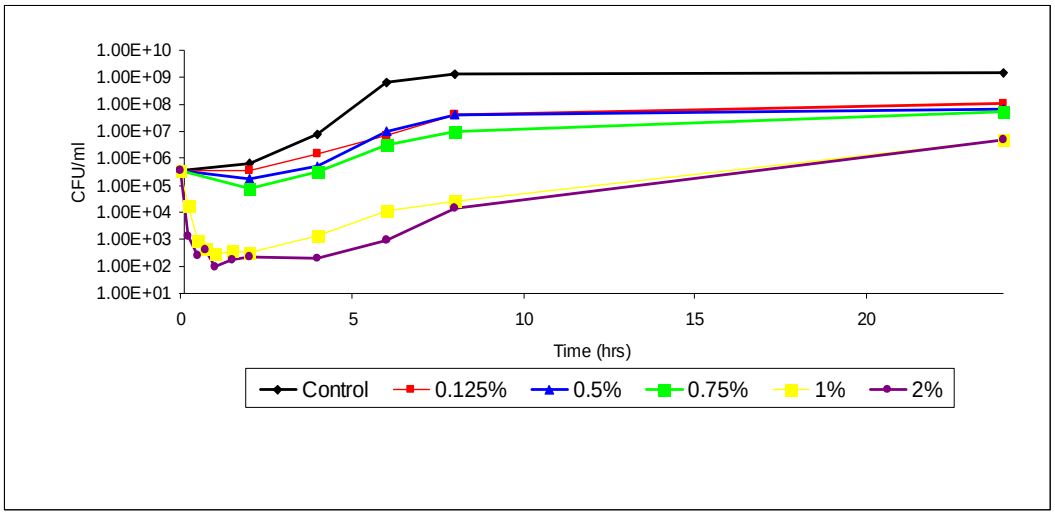


Figure 2.9 Time-kill expressed in Log_{10} reduction of *P. aeruginosa* exposed to *M. flabellifolius* essential oil over 24 hr.

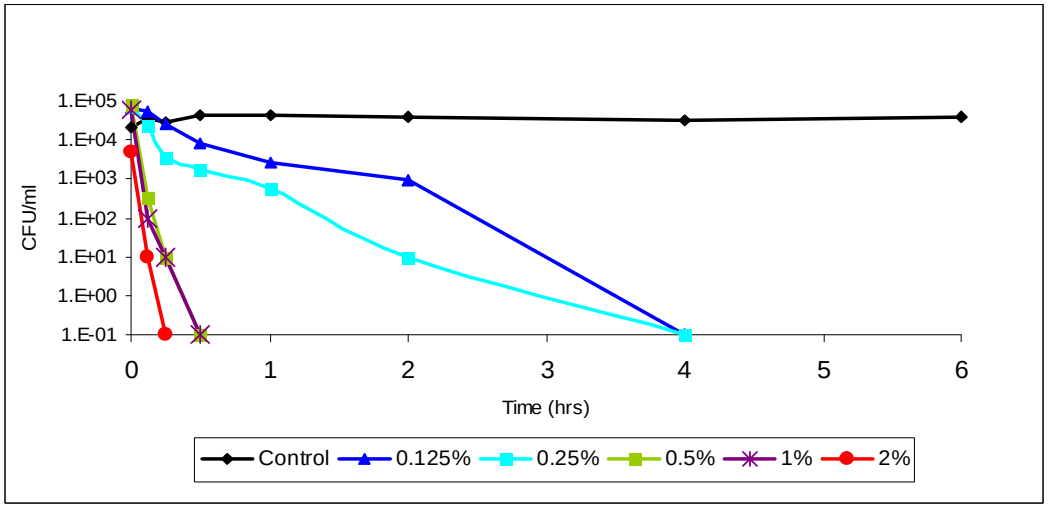


Figure 2.10 Time-kill expressed in Log_{10} reduction units of *C. albicans* exposed to *M. flabellifolius* essential oil within the first six hours.

While it is not always the major essential oil constituents responsible for antimicrobial activity (Chalchat and Garry, 1997), it is however, interesting to tentatively correlate the observed antimicrobial activity to the essential oil composition for *M. flabellifolius*. Pinocarvone, a major constituent (11.13%) in *M. flabellifolius* essential oil is also one of the major constituents of Hyssop oil (*Hyssopus officinalis*), which is a popular home-remedy with bactericidal properties (Kerrola *et al*., 1994). Hinou *et al.* (1989) reported that *trans*-isomers have higher activities than the corresponding *cis*-isomers. *trans*-Pinocarveol and oxygenated bicyclic terpenoid, pinocarvone present in *M. flabellifolius* are both *trans*-isomers, possibly contributing to the

antimicrobial activities seen here. Kishore *et al*. (1996), found the essential oil of *Chenopodium ambrosioides* with *trans*-pinocarveol as a major constituent (27%) to be fungicidal against several dermatophytes. The high fungicidal activities noted in the disc diffusion assay (5-10 mm) for *M. flabellifolius* could possibly be attributed to the high concentration of *trans*-pinocarveol (19.57%). It is worthy of note that *trans*-pinocarveol, the major essential oil component in *M. flabellifolius*, is used in pharmaceutical preparations such as Ozopulmin^{TM} to treat respiratory tract disorders, including asthma. The antimicrobial properties, with speculation on possible structure activity relationships reported here for *M. flabellifolius* essential oil, may provide some pharmaceutical rationale for the popular traditional use of this plant in African medicinal herbal preparations.

2.7 General conclusions

- The major constituents of the essential oil of *Myrothamnus flabellifolius* are *trans*-pinocarveol (19.6%) and pinocarvone (11.1%), representing 30.7% accumulatively.

- The highest antimicrobial activities obtained by means of the disc diffusion assay were noted for the fungal organisms *Cryptococcus neoformans* (8 mm) and *Aspergillus niger* (10 mm).

- The highest antimicrobial activity noted in the MIC assay was also for *Cryptococcus neoformans* (2 mg/mL).

- Death kinetics for the three organisms studied demonstrated that the killing rate was greatest for *Candida albicans*, then *Staphylococcus aureus*, and lastly only initial activity for *Pseudomonas aeruginosa* with regrowth after two hours.

- The ethnobotanical use of *Myrothamnus flabellifolius* to treat respiratory ailments correlates with the high antimicrobial activities found for *Cryptococcus neoformans* in the disc diffusion and MIC assay.

Chapter 3

Osmitopsis asteriscoides (P.J. Bergius) Less., the antimicrobial efficacy (disc diffusion, minimum inhibitory concentration, time-kill) and role of the major essential oil constituents.

3.1 Introduction

Osmitopsis asteriscoides (P.J. Bergius) Less., (Asteraceae) is known locally as 'bels' or 'belskruie'. The plant is known for its abundance of essential oil which imparts a camphorus odour (Watt and Breyer-Brandwijk, 1962). Previous phytochemical work has been undertaken on *Osmitopsis* (Bohlmann and Zdero, 1974 in van Wyk *et al*., 1997; Bohlmann *et al*., 1985) which reported the presence of sesquiterpene lactones. More recently (Scott *et al*., 2004) described the morphology and phytochemical profile of *O. asteriscoides* extracts. Microbiological activity was investigated but this was restricted to the disc diffusion assay on aqueous extracts. This Chapter focuses on the microbiological activity and composition of the essential oil, which is the most characteristic feature of this highly aromatic plant. Although the antimicrobial activity of essential oils and their constituents are well-known (Hinou *et al*., 1989; Yousef and Tawil, 1980; Pattnaik *et al*., 1997; Hammer *et al*., 1999) the methods used to assess the *in vitro* antimicrobial action remains a topic of debate. This study has also documented the variability, reproducibility and accuracy in the results obtained using different methods (disc diffusion, MIC, time-kill and membrane confocal integrity studies). Furthermore the interaction between major compounds was assessed for antimicrobial activity.

3.2 Botanical description

The leaves of the plant have numerous surface glands containing essential oil and when brushed the plant emits a strong eucalyptus-camphor smell. The plant is characterised by large thin, leaves and daisy-like white flowers as seen in Figure 3.1 (van Wyk *et al*., 1997).



Figure 3.1 The leaves and flowers of *Osmitopsis asteriscoides.*

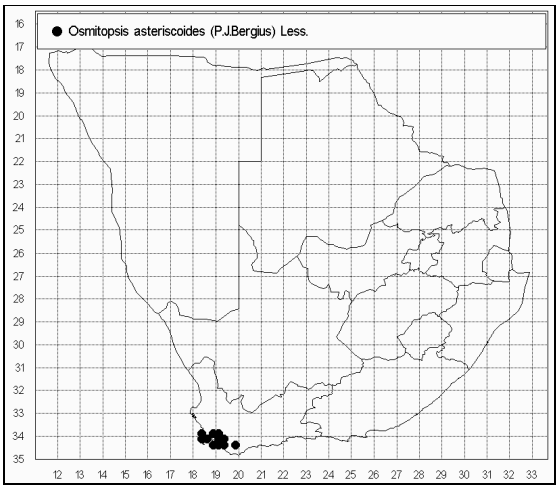


Figure 3.2 The geographical distribution of *Osmitopsis asteriscoides* in South Africa SANBI).

3.3 Distribution

Osmitopsis asteriscoides is restricted to the South Western Cape region (Figure 3.2) in South Africa (Bremer, 1972).

3.4 Medicinal uses

This Cape-Dutch remedy has been traditionally used to treat various ailments and may be taken orally in the form of a brandy tincture for chest complaints, bronchial congestion and other respiratory related illnesses (Watt and Breyer-Brandwijk, 1962; Scott *et al.*, 2004). Furthermore, *O. asteriscoides* has been combined with other plant species i.e. *Artemisia afra* and *Eucalyptus globulus* to treat respiratory infections (Watt and Breyer-Brandwijk, 1962). The dried plant may also be used externally to treat inflammation, cuts and swelling (van Wyk *et al*., 1997). The use of *O. asteriscoides* for intestinal disorders, stomach complaints and fever has also been reported (Watt and Breyer-Brandwijk, 1962).

3.5 Methods

3.5.1 *Chemical aspects*

Plant collection and distillation of essential oils: The aerial parts of the plants were collected in the growing season (February) from a natural population near Betty's Bay, South Western Cape region, South Africa. A voucher specimen (Table 3.1) is retained in the Department of Pharmacy and Pharmacology, University of Witwatersrand. The plant material was distilled as described in Chapter 2.5.1.

Table 3.1 Plant collection data for *O. asteriscoides.*

Plant	Voucher	Material distilled (g)	Essential oil yield (% w/w)
O. asteriscoides	ADCAV174	2520.0	0.7

Gas chromatography combined with mass spectrometry (GC-MS): Oil samples were analyzed with GC-MS using the Hewlett-Packard 1800A GCD system operating under the same conditions as described in Chapter 2.5.1. The chiral separation of camphor was performed on a multidimensional gas chromatography / mass spectrometer (MD-GC/MS) system. Two Hewlett Packard GC 6890 systems with MSD and Gerstel multi column switching (MCS) system were used. The cooled injection system (CIS) was kept at 40°C for injection. Helium was used as carrier gas (1 mL/min). The precolumn comprised of an HP-Innowax fused silica capillary column (60 m x 0.25 mm i.d., with 0.25 um film thickness). The GC oven temperature was kept at 60 °C for 10 min and programmed to 220 °C at a rate of 4 °C/min and kept constant at 220 °C for 10 min, then programmed to 240 °C at a rate of 1 °C/min and kept constant at 240 °C for 40 min. The FID detector temperature was at 250 °C. The main column comprised: Lipodex E [Octakis (3-*O*-butyryl-2, 6-di-*O*-pentyl)-g-cyclodextrin] (70% in OV 1701), (25m x 0.25 mm i.d.). The temperature programme for camphor was 40 °C for 34 min and programmed to 120 °C at a rate of 1 °C/min then kept constant at 120 °C for 6 min. The MS were taken at 70 eV. Mass range was from m/z 35 to 425.

3.5.2 Antimicrobial aspects

Culture and media preparation were undertaken according to the NCCLS (2003) guidelines and additional references as described in Chapter 2.5.2. Three different antimicrobial assays (disc diffusion, minimum inhibitory concentrations and time-kill studies) were performed on the hydrodistilled oil to corroborate results.

Disc diffusion assays: A broad disc diffusion screening was carried out against many of the same reference pathogens as listed in Table 2.2. Some variation in the selection of pathogens was undertaken. Two additional Gram-positive organisms were added to the study i.e. *Bacillus subtilis* (ATCC 15244) and *Staphylococcus epidermidis* (ATCC 2223). Rabe and van Staden (1998) and authors Mangena and Muyima (1999) have shown a better efficacy towards Gram-positives due to membrane structure and this warranted their inclusion in the study. *Staphylococcus epidermidis* was included in the study due to its pathogenesis being associated with immunocompromised patients, causing severe nosocomial infections such as endocarditis. This opportunistic pathogen resides on the skin as part of the normal biological flora, but in times of stress or reduced immunity it can cause a number of infections (Bannister *et al*., 2000). The disc diffusion method is described in Chapter 2.5.2.

Minimum inhibitory concentrations (MIC): The microplate bioassay minimum inhibitory concentrations were determined using the micro-dilution method (Carson *et al*., 1995; Eloff, 1998a; NCCLS, 2003) as described in Chapter 2.5.2. Table 3.2 gives the acceptable control ranges for ciprofloxacin and amphotericin B against additional pathogens used in the MIC assays. Where pathogen strain numbers were the same as that used in Chapter 2, refer to Table 2.2.

Table 3.2 Acceptable MIC ranges for ciprofloxacin antibiotic control.

Test organism	MIC controls (μg/mL)*
Escherichia coli (ATCC 11775)	0.04-0.10
Staphylococcus epidermidis (ATCC 2223)	0.30-2.50
Bacillus subtilis (ATCC 6051)	0.09-3.10

* Acceptable ranges as determined within the Department of Pharmacy and Pharmacology, University of Witwatersrand (controls in this study were run for all test organisms).

Minimum inhibitory assays were done in triplicate and the mean tabulated (Table 3.4). A 0.4 mg/mL *p*-iodonitrotetrazolium violet (Sigma-Aldrich) solution (INT) was prepared and 40 µl transferred to all inoculated wells. The microtitre plates inoculated with bacteria were examined after six hours to determine a colour change in relation to concentration of microbial growth. The yeasts were examined after 24 hr.

Time-kill methodology: Time-kill assays were employed according to methods as described in Chapter 2.5.2 investigating the pathogens *S. aureus* (Gram-positive), *P. aeruginosa* (Gram-negative) and a yeast *C. albicans.* Oil concentrations of 0.5, 1, 1.5 and 2% (5.2, 9.5, 13.8 and 18.1 mg/mL respectively) were selected to determine the death kinetics for *O. asteriscoides.* As with the *M. flabellifolius* study (Chapter 2), results were corroborated in a separate laboratory at the University of Iowa (Viljoen *et al*., 2003).

Membrane integrity: Harvesting of bacterial cells, treatment and confocal scanning for the assessment of membrane integrity was undertaken according to standard methods described by Lindsay and von Holy (1999) and Peta *et al*. (2003). Stock cultures of *S. aureus* were grown in Tryptone Soya (Oxoid) broth and centrifuged (MSE) for 10 min at 5000 rpm. The supernatants were discarded and the pellets resuspended in 10 mL of a 0.9% NaCl (Labchem) solution with 50 µl Tween 80 (Saarchem). *Osmitopsis asteriscoides* oil at 0.5% was added to the buffer. A control having no essential oil was run in tandem. At time intervals comparable to the time-kill assay, 1 mL aliquots were removed and added to 9 mL Neutralizing Buffer (Difco). After centrifugation for 10 min at 5000 rpm, samples were washed with 1 mL of a 0.9% NaCl solution and recentrifuged. The supernatants were discarded and the pellet resuspended in 100 µl of a 0.9% NaCl solution. Samples (0.5 % *O. asteriscoides* oil and control) were prepared for examination using the LIVE / DEAD® *Bac*light Bacterial Viability Kit (Molecular Probes) and thereafter viewed using a confocal scanning electron microscope (Carl Zeiss). The morphology of cells exposed to the oil were compared with those untreated control cells by observing the interaction of the dyes with bacterial cells. Bacteria with intact cell membranes stained fluorescent green and bacteria having damaged or compromised membranes stained orange-red. Experimental procedures were repeated for conformity.

Major compound investigation: The compounds 1,8-cineol at 98.0% purity (Lot 1054365, Sigma-Aldrich), (+)-camphor at 98.0% purity (Lot 464-48-3, Sigma-Aldrich) and (-)-camphor at 99.0% purity (Lot 464-48-2, Sigma-Aldrich) were investigated for the role they may play on the

anti-candidal activity of the essential oil. Initial MIC determination was undertaken as a screening process to investigate the activity of the individual compounds. Assays were done in triplicate and the mean tabulated (Table 3.5). Individual compounds were prepared at a starting concentration of 128 mg/mL and assayed with the essential oil of *O. asteriscoides*. A more detailed assessment of the role on the two main chemical components i.e. 1,8-cineole and camphor was undertaken by comparative time-kill studies where *C. albicans*, a moderately susceptible test organism, was used to demonstrate the efficacy of 1,8-cineole and camphor independently and in combination in concentrations relative to their ratio in the plant over a 24 hr period. Time-kill studies were done in duplicate and the mean plotted in Figure 3.8.

3.6 Results and discussion

3.6.1 Essential oil chemistry

Twenty-five compounds were identified in the essential oil of *O. asteriscoides* representing 96.1% of the total composition (Table 3.3).

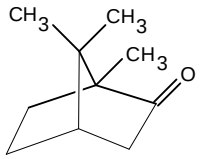
Table 3.3 Essential oil composition of *O. asteriscoides*.

RRI*	Compound name	%
1014	tricyclene	tr
1032	α-pinene	3.0
1035	α-thujene	0.1
1076	camphene	1.8
1118	ß-pinene	0.6
1132	sabinene	1.4
1174	myrcene	tr
1188	α-terpinene	tr
1195	dehydro-1,8-cineole	0.3
1203	limonene	tr
1213	**1,8-cineole**	**59.9**
1255	γ-terpinene	0.3
1280	*p*-cymene	0.9
1290	terpinolene	tr
1391	(*Z*)-3-hexen-1-ol	tr
1450	*trans*-linalool oxide (furanoid)	tr
1474	*trans*-sabinene hydrate	0.1
1482	longipinene	2.9
1493	α-ylangene	tr
1499	α-campholenal	tr
1522	chrysanthenone	tr
1532	**camphor, (-)**	**12.4**

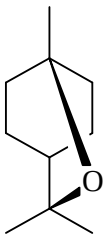
RRI*	Compound name	%
1553	linalool	0.3
1556	*cis*-sabinene hydrate	0.1
1571	*trans*-p-menth-2-en-1-ol	0.1
1586	pinocarvone	0.1
1611	terpinen-4-ol	2.3
1638	*cis*-p-menth-2-en-1-ol	tr
1648	myrtenal	tr
1651	sabinaketone	tr
1664	*trans*-pinocarveol	0.1
1682	δ-terpineol	0.4
1706	α-terpineol	7.8
1758	*cis*-piperitol	tr
1798	methyl salicylate	0.1
1804	myrtenol	tr
1864	*p*-cymen-8-ol	tr
2008	caryophyllene oxide	0.4
2008	p-menta-1,8-dien-10-ol	0.1
2113	cumin alcohol	tr
2265	longiverbenone (= vulgarone B)	0.2
	TOTAL	**96.1**

*RRI: Relative retention indices calculated against n-alkanes
% calculated from TIC data
tr = trace (< 0.1%)

Major compounds: The two major compounds (Figure 3.3) in the essential oil of *O. asteriscoides* are 1,8-cineole (59.9%) and (-)-camphor (12.4%), representing 72.3% accumulatively.



(-)-camphor



1,8-cineole

Figure 3.3 Chemical structures for major compounds identified in the essential oil of *O. asteriscoides*.

3.6.2 Antimicrobial activity

Of the twelve test organisms studied in the disc diffusion assay, four (*P. aeruginosa*, *B. subtilis*, *A. niger* and *A. alternata*) showed no inhibitory effect. The highest activities were obtained for the yeasts (*C. neoformans*, 3 mm and *C. albicans*, 2 mm) and *S. aureus* (3 mm). For the MIC assay highest sensitivities were noted for *E. coli* and *S. epidermidis* (4 mg/mL), followed by moderate sensitivity (8 mg/mL) for the three Gram-positive organisms *S. aureus, E. faecalis, B. subtilis* and the yeasts *C. albicans* and *C. neoformans.* Table 3.4 lists the zones of inhibition (mm) and MIC values (mg/mL) for the test organisms studied. The MIC values recorded here do not always correlate with previously published data (Viljoen *et al*., 2003). Variation may be attributed to the mean been calculated with a further range of reference cultures and more repetitions as the techniques were optimised. The correlation between the two different screening methods was examined and larger zones of inhibition did not always correlate with lower MIC values. Variations occurred for *P. aeruginosa* where no activity was noted in the disc diffusion assay but in the MIC assay some minor activity (16 mg/mL) was noted. This variation is more clearly seen in the time-kill assay (Figure 3.5) where a steady progression of antimicrobial action is noted over 24 hr. This trend is also evident with the test organism *B. subtilis* where antimicrobial activity (8 mg/mL) is only noted in the MIC assay. Possible regrowth could have occurred (not visible in the MIC assay and in the disc diffusion assay) due to the presence of resistant spores. *Escherichia coli* (4 mg/mL, 1.5 mm) and *S. epidermidis* (4 mg/mL, 1 mm) indicated MIC values lower than what would be expected when observing / correlating with inhibition zones. This variation between methods was also noted by Pattnaik *et al.* (1996), Chalchat and Garry (1997), Janssen *et al.* (1987), Pauli and Kubeczka (1997) and Remmal and Tanaoui-Elaraki (1993) and can be attributed mainly to the variation of neat essential oil on disc, disc size, agar composition as well as the volatility of oil in an open air system. The incubation period for the moulds are seven days and the volatility of the essential oils over this extended incubation period may be responsible for the negative inhibition results obtained in the disc diffusion assay.

Table 3.4 Disc diffusion and MIC assays of *O. asteriscoides* essential oil.

Test organism	Disc diffusion (mm) [*1]	Disc diffusion controls[*2]	MIC (mg/mL)	MIC controls[*4]
Pseudomonas aeruginosa ATCC 9027	0	2	16	2.5×10^{-3}
Escherichia coli ATCC 11775	2	10	4	0.08×10^{-4}
Salmonella typhimurium ATCC 14028	1	8	32	0.8×10^{-3}
Proteus vulgaris (clinical strain)	1	12	16	1.6×10^{-4}
Staphylococcus aureus (ATCC 25923)	3	14	8	0.5×10^{-3}
Staphylococcus epidermidis ATCC 2223	1	2	4	2.5×10^{-3}
Enterococcus faecalis ATCC 29212	1	8	8	2.0×10^{-3}
Bacillus subtilis ATCC 6051	0	10	8	2.5×10^{-3}
Cryptococcus neoformans ATCC 90112	3	16	8	2.5×10^{-3}
Candida albicans ATCC 10231	2	14	8	1.3×10^{-3}
Aspergillus niger (clinical strain)	0	20	ND[*3]	ND[*3]
Alternaria alternata (clinical strain)	0	4	ND[*3]	ND[*3]

[*1] The diameter of the zone of inhibition is expressed in mm from the disc edge.
[*2] Neomycin and nystatin served as controls for bacteria and fungi respectively.
[*3] ND = Methods did not incorporate MIC's for moulds.
[*4] Ciprofloxacin and amphotericin B served as controls for bacteria and yeasts respectively.

Results of the time-kill study are presented in Figures 3.4 to 3.6. Positive controls (ciprofloxacin and amphotericin B) presented with an immediate death response and due to presentation scale were not included in the time-kill plots. As with the *M. flabellifolius* study, results duplicated in an independent laboratory in a collaborative investigation with Prof. Klepser were mainly congruent. Further comparative assessment can be observed in Viljoen *et al.* (2003). The cidal activity was exhibited for concentrations ranging between 0.5% and 2.0% over 24 hr. For the *S. aureus* (ATCC 29523) time-kill plot, the essential oil generally followed a concentration-

dependent antibacterial activity (Figure 3.4). There is an initial decrease in viable counts for all concentrations within the first 60 min after which the 1.5% (13.8 mg/mL) and 2% (18.1 mg/mL) oil concentrations show increasing bactericidal activity with time where cidal activity is evident after 24 hr. At concentrations of 0.5% (5.2 mg/mL) and 1% (8.1 mg/mL) a bacteriostatic effect was noted after eight hours with viable counts neither increasing nor decreasing after 24 hr. These results correlate with the MIC values (8 mg/mL) obtained for *S. aureus*. In Figure 3.5 the death kinetics show a decrease in the number of surviving *P. aeruginosa* cells within 60 min, but an increase in colony forming units over time indicates that, after an initial bacteriostatic effect, regrowth of the organism occurs. The 24 hr time-kill plot (Figure 3.5) for *P. aeruginosa* shows a more detailed progression of bacteriostatic activity, which can obviously not be recorded by the disc diffusion method. The regrowth effect gives some rationale for the differences of results obtained for the disc diffusion assay where no activity was recorded. Some activity (8 mg/mL), congruent with the time-kill assays was noted for the MIC assay. Figure 3.6 displays the cidal effect of the essential oil on *C. albicans* (ATCC 10231). The candidacidal activity is rapidly exhibited and viable counts were considerably lowered within 240 minutes for all the concentrations and then increasing cidal action with increasing oil concentration. At 24 hr complete fungicidal activity was exhibited for all oil concentrations. The candidacidal activity reported here could possibly provide scientific support for the traditional use of *O. asteriscoides*.

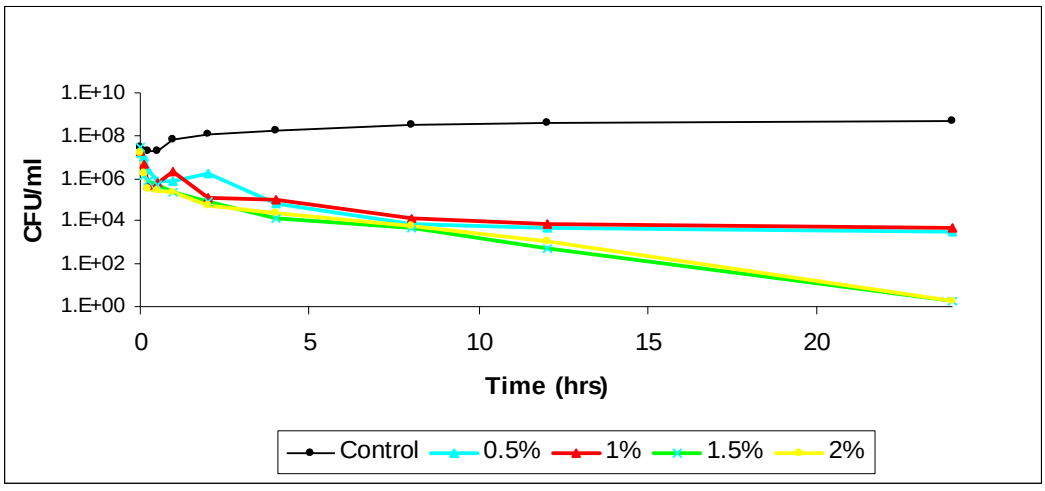


Figure 3.4 Death kinetic studies of *S. aureus* with exposure to the essential oil of *O. asteriscoides* as seen over a 24 hour period.

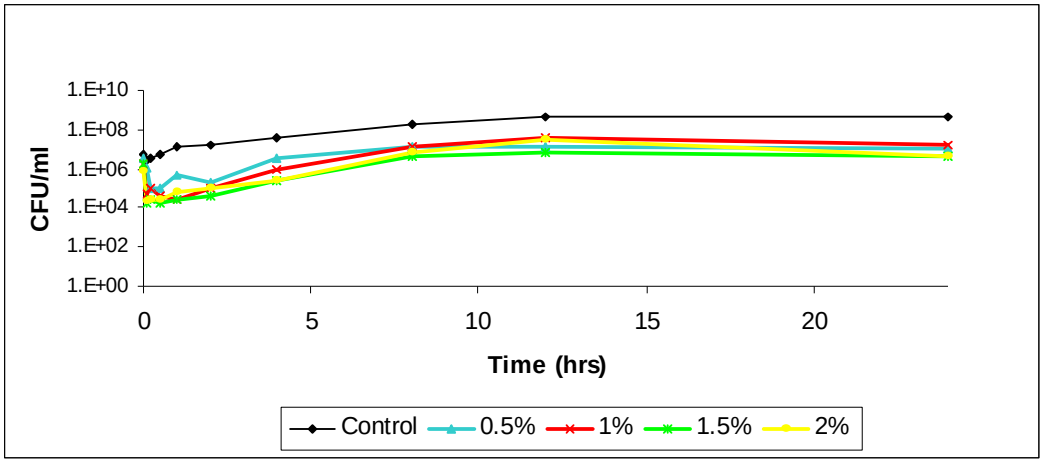


Figure 3.5 Death kinetic studies of *P. aeruginosa* with exposure to the essential oil of *O. asteriscoides* as seen over a 24 hour period.

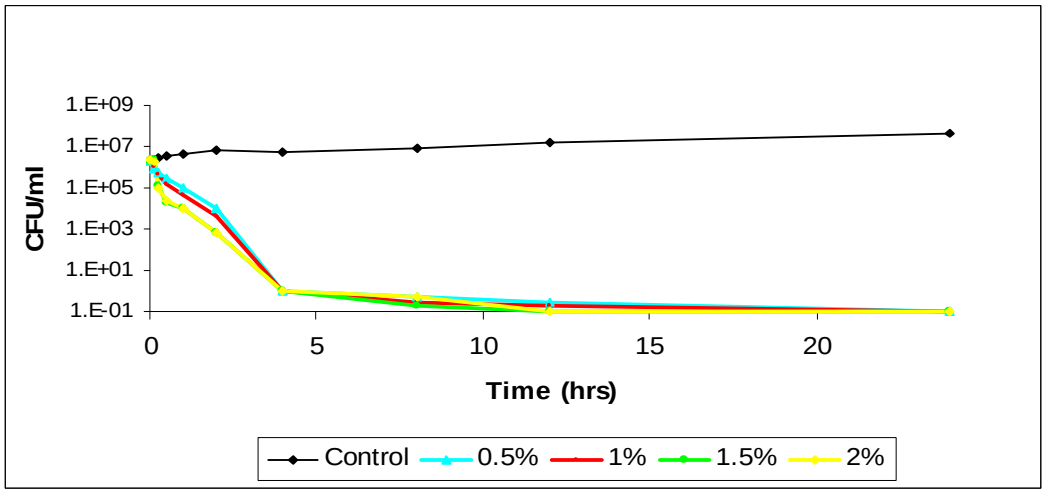


Figure 3.6 Death kinetic studies of *C. albicans* with exposure to the essential oil of *O. asteriscoides* as seen over a 24 hour period.

Comparison of the time-kill plots for the three organisms studied showed that the killing rate was the greatest for *C. albicans*, then *S. aureus* and then with initial activity followed by subsequent regrowth for *P. aeruginosa*. For *S. aureus*, steady killing rates were obtained at 2% essential oil concentration. With *P. aeruginosa*, regrowth before four hours was noted for all concentrations with growth reduction for the initial 60 min. For *C. albicans*, a concentration dependent cidal activity was noted with greatest cidal activity at 2% after 12 hr.

Membrane integrity: In conjunction with time-kill analysis, a membrane integrity study was undertaken on 0.5% *O. asteriscoides* essential oil sample. This method correlated very well with the time-kill studies. Figure 3.7 shows a proportion of the confocal image viewed and selected

for cell counting. This view of the *S. aureus* cells when exposed to 0.5% oil of *O. asteriscoides* indicate viability (green cells) seen for most cells in the control (0-20 hr) and on initial exposure to the oil. There is some cell death as this is acceptable in growing cell populations. Over time, however, the 0.5% *O. asteriscoides* oil sample show increasing cell death (orange-red). In comparison with the time-kill showing the kinetics for 0.5% *O. asteriscoides* after five hours (Figure 3.7) results were comparable. The area above the line after five hours depicts viable cells. Below the line shows non-viability, indicative of a bacteriostatic action or cell injury. This corresponds with the data generated in the confocal membrane integrity study (Table 3.5) also having injured populations with live and dead cells in the same proportion. In addition, membrane integrity studies display cell lysis which is some indication of how the essential oil is working to inactivate microbial cells.

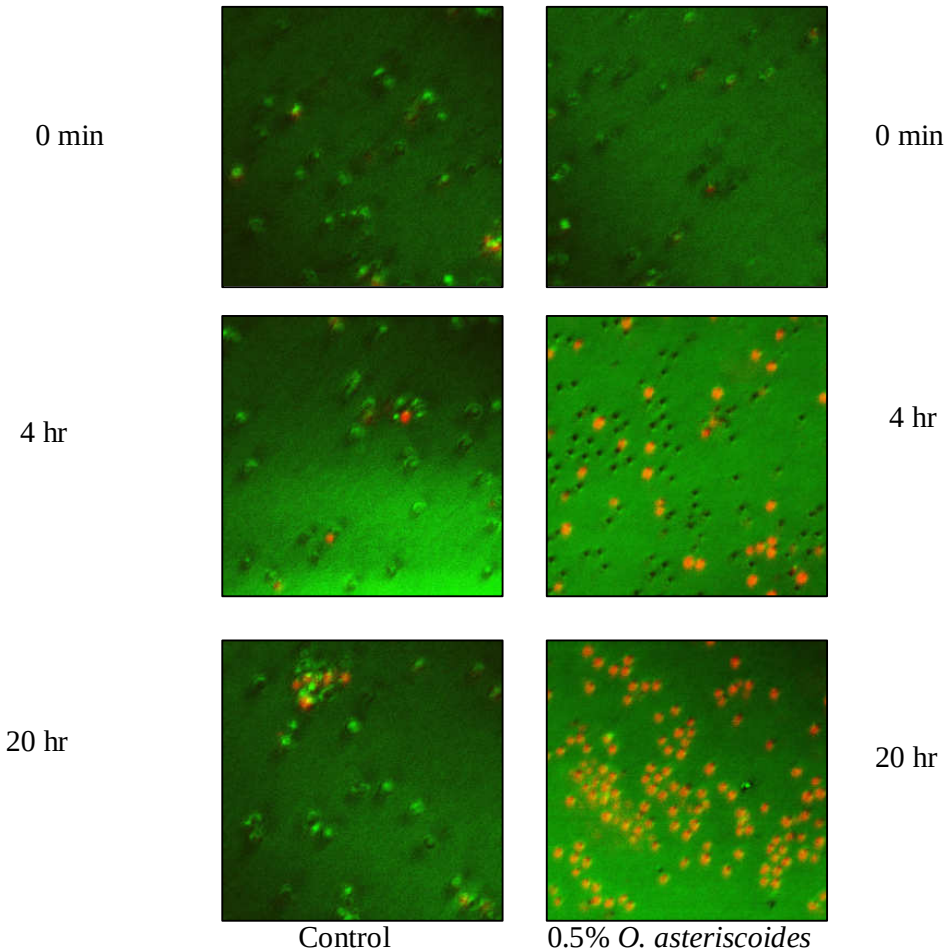


Figure 3.7 The confocal scanner laser images (2 µm) of the control (having no essential oil) and 0.5% *O. asteriscoides* over 24 hr.

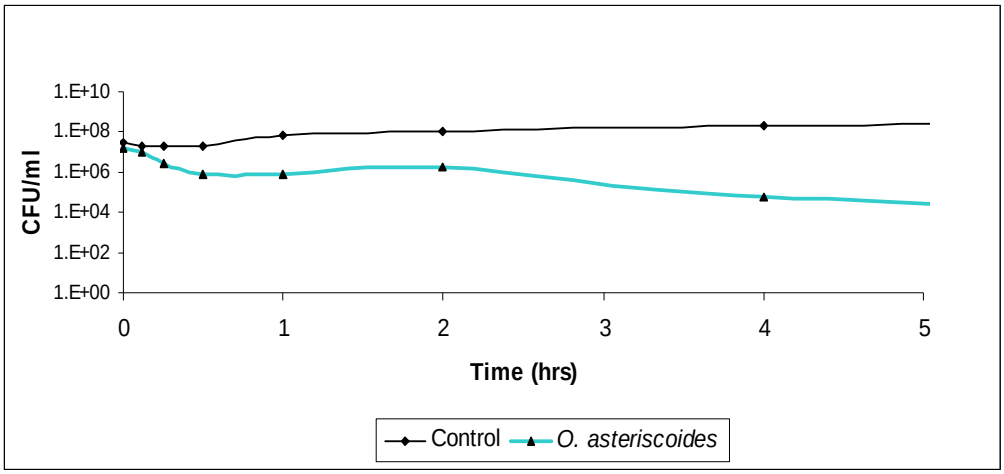


Figure 3.8 Death kinetic studies of *S. aureus* with exposure to the essential oil (0.5%) of *O. asteriscoides* over five hours.

Table 3.5 The confocal cell viability (%) of *S. aureus* cells when exposed to 0.5% *O. asteriscoides* over 4 hr and 20 min.

Time	Sample	Cell viability (%)		
		Live	Dead	Injured
0 min	Control	92.6	7.4	0
	0.5% *O. asteriscoides*	79.1	20.2	0.7
30 min	Control	97.6	2.4	0
	0.5% *O. asteriscoides*	49.3	34.9	15.8
2 hr, 15 min	control	95	4.7	0.3
	0.5% *O. asteriscoides*	36.4	57.3	6.3
4 hr, 20 min	Control	93.9	4.5	1.6
	0.5% *O. asteriscoides*	20.2	20.2	59.6

Major compound investigation: Further studies on the role of the two major chemical components 1,8-cineole (60%) and (-)-camphor (12%) on the antimicrobial activity of *C. albicans* was demonstrated with MIC's and death kinetic studies. Previous MIC studies on the compounds 1,8-cineole and camphor have shown conflicting results when tested against *C. albicans*. Magiatis *et al.* (2002), indicated moderate activity when tested against camphor (6.2 mg/mL) and good activity (0.3 mg/mL) when tested against 1,8-cineole. Pattnaik *et al.* (1997) reported candidal resistance when tested against 1,8-cineole. Setzer *et al.* (2004) indicated good activity for *C. albicans* when tested against both 1,8-cineole and camphor (0.6 mg/mL). Griffin *et al.* (1999), reported moderate candidal activity for 1,8-cineole with camphor being more

active. Contradictory reports may be due to tests been undertaken on different *C. albicans* reference strains or differentiation in compound purity. From the MIC data seen in Table 3.6, it can be noted that 1,8-cineole (16 mg/mL), (+)-camphor (32 mg/mL) and (-)-camphor (16 mg/mL) are less active having MIC values higher than that of *O. asteriscoides* (8 mg/mL). When the combination of (-)-camphor with 1,8-cineole was tested against *C. albicans*, a significantly lower MIC value (0.5 mg/mL) was obtained. This indicates that a combination of these two major compounds may be responsible for the increased activity found in *O. asteriscoides*. Even though some research has been carried out on essential oil combinations (Geda, 1995; Lachowicz *et al.*, 1998), investigation of the synergistic role of constituents have been neglected.

Table 3.6 The MIC of *O. asteriscoides* essential oil and major constituents against *C. albicans.*

Sample	MIC (mg/mL)
O. asteriscoides	8.0
1,8-cineole	16.0
(+)-camphor	32.0
(-)-camphor	16.0
1:1 (-)-camphor with 1,8-cineole	0.5
nystatin control	1.3×10^{-3}

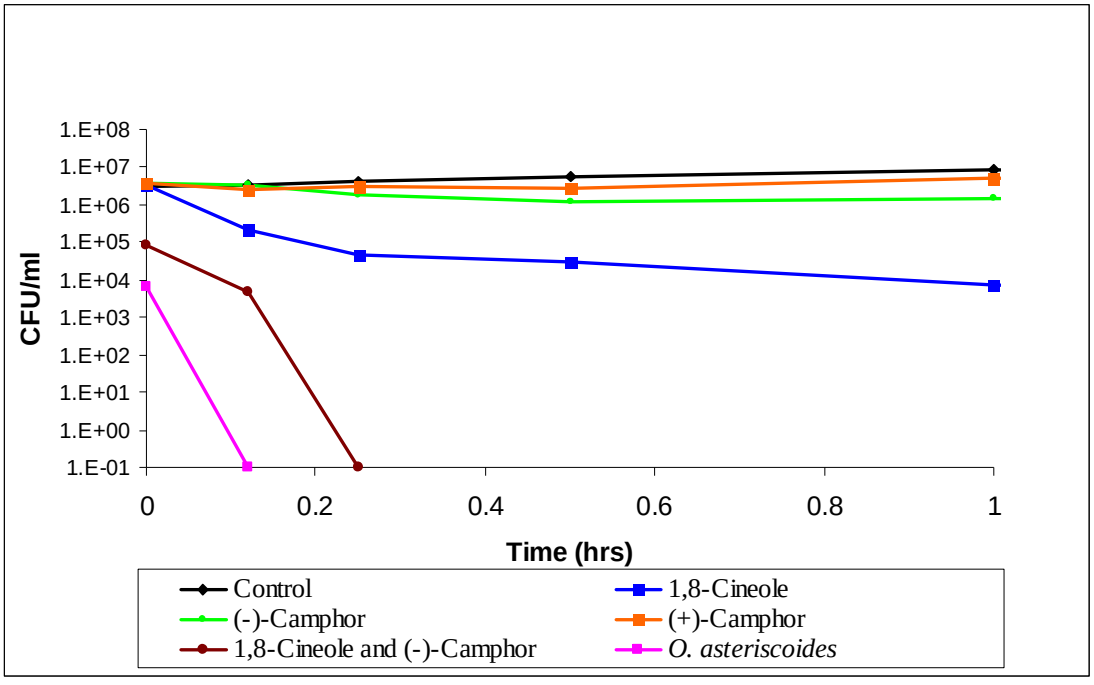


Figure 3.9 Time-kill plot for *O. asteriscoides* essential oil (1%), 1,8-cineole, (+)-camphor,

In order to investigate this further, a time-kill assay (Figure 3.9) was undertaken and results show that both (+)-camphor, (-)-camphor have negligible antimicrobial activity on *C. albicans* whereas 1,8-cineole indicated microbial reduction after 240 min. As *O. asteriscoides* only accumulates levorotatory camphor, it was tested in combination with 1,8-cineole and a total reduction of colony forming units was observed within 15 min. This indicates that the positive antimicrobial response was due to possible synergistic interaction between 1,8-cineole and (-)-camphor. From Figure 3.7, it is evident that some minor compounds should also be taken into consideration as 1% essential oil still had a greater killing rate (under 10 min) than 1,8-cineole and (-)-camphor in combination.

Camphor is known to be a decongestant and antiseptic (Bruneton, 1995) and it is further known for its topical use as a counter-irritant in fibrositis (Reynolds, 1996). The enantiomeric composition of camphor has been discussed by Demirci *et al.* (2002) where reference was made to 40 different essential oil samples with (-)-camphor occurring more abundantly than (+)-camphor. Nakatsu *et al.* (2000), reported inactivity of 1,8-cineole against Gram-positive bacteria, however, with *S. aureus*, relatively moderate activity (Table 3.4) for *O. asteriscoides* was noted. Cineole-rich essential oils have been used historically for respiratory infections (Silvestre *et al.*, 1999). Previous studies by Pattnaik *et al.* (1997), show that 1,8-cineole at 23.2% has an antimicrobial effect against 18 microbial strains, with the exception of *P. aeruginosa* which showed no inhibitory effect. In antimicrobial studies done on *O. asteriscoides* having 60% 1,8-cineole, no lasting antimicrobial effect was noted with *P. aeruginosa*. The antifungal activity of 1,8-cineole, specifically with *C. albicans* was also noted by Steinmetz *et al.* (1988) where a decrease of fungal activity was reported after six hours for rosemary essential oil (composition = 51% 1,8-cineole and 11% camphor). Similarly, with *O. asteriscoides*, there was a 90% reduction in fungal activity after six hours. *Jasonia montana*, another commonly used folk medicine, having an essential oil composition of 21.79% of camphor and 4.6% 1,8-cineole was reported by Hammerschmidt *et al.* (1993) to have antifungal activity against *C. albicans* and *C. neoformans*. This corroborates the findings summarised in Table 3.4. In a plant screening by Tzakou *et al.* (2001) of *Salvia ringens* containing concentrations of 1,8-cineole (46.4-50.7%), comparable to that obtained for *Osmitopsis asteriscoides* no activity was reported for *Staphylococcus aureus* and *Staphylococcus epidermidis*, but very good activity for the Gram-negatives. Results of *O. asteriscoides* show that the oil is active against *S. aureus* and *S. epidermidis*. It was thought to be due to the high concentration of camphor (14.9%). Also of note was the fact that camphor was only present in small quantities (1.6-1.8%) for *Salvia ringens* (Tzakou *et al.*, 2001).

Previous phytochemical work has been undertaken on *Osmitopsis* (Bohlmann and Zdero, 1974; Bohlmann *et al.*, 1985) who reported the presence of sesquiterpene lactones. These compounds are known for their wide range of biological activity (Bruneton, 1995) and the monoterpenes, contained in the essential oil, especially 1,8-cineole and (-)-camphor, provides a chemical rationale for the established traditional use of *O. asteriscoides* as a Cape-Dutch remedy, which is still widely used today.

3.7 General conclusions

- The major constituents of the essential oil of *Osmitopsis asteriscoides* are 1,8-cineole (59.9%) and (-)-camphor (12.4%), representing 72.3% accumulatively.

- The highest antimicrobial activities obtained by means of the disc diffusion assay were noted for the fungal organism *Cryptococcus neoformans* (3 mm) and *Staphylococcus aureus* (3 mm).

- The highest antimicrobial activity noted in the MIC assay was against *Staphylococcus epidermidis* (4 mg/mL) and *Escherichia coli* (4 mg/mL).

- Death kinetics for the three organisms studied demonstrated that the killing rate was greatest for *Candida albicans*, then *Staphylococcus aureus*, and lastly only initial activity for *Pseudomonas aeruginosa* with regrowth after two hours.

- The ethnobotanical use of *Osmitopsis asteriscoides* to treat dermal afflictions correlates with the highest antimicrobial activities found for the *Staphylococci*.

- Method corroboration was achieved between different laboratories (Universities Iowa and Witwatersrand) for the time-kill assay and between methods (MIC / time-kill and time-kill / confocal integrity studies).

- The two major essential oil constituents (1,8-cineole and (-)-camphor) act synergistically to enhance antimicrobial activity.

Chapter 4

The antimicrobial activity of *Artemisia afra* Jacq. ex Willd essential oil and the role of the major constituents, singularly and in combination.

4.1 Introduction

The genus *Artemisia* (Asteraceae) is one of the most well-known medicinal plants and has been used globally to treat infectious diseases (Setzer *et al*., 2004). Its reputation in medicine dates back to the 15th century when some species e.g. *A. absinthium* were not only used as an antiseptic but also incorporated into alcoholic beverages and used in folklore celebrations as love charms. The word "Artemisia" is derived from the Greek goddess Artemis. Of the 180 species in this genus, *Artemisia afra* Jacq. ex Willd is the only indigenous species (*afra* means Africa) and is one of the most widely used medicinal plants (Graven *et al*., 1992). Its popularity may be attributed not only to the wide use as a treatment for mainly respiratory ailments but also its vast abundance in the wild. A recent study on the use of medicinal plants in the Bredasdorp region of South Africa confirmed its popularity when a survey revealed that of the 44 participants interviewed, all reported the medicinal use of *A. afra*, making it one of the most popular plants used by the local people (Thring and Weitz, 2006). In spite of the international interest this genus has generated both on a commercial market and within indigenous populations, very little quantitative microbiological analysis has been undertaken. Some literature (Graven *et al*., 1992; Gundidza *et al*., 1993; Mangena and Muyima, 1999; Huffman *et al.*, 2002; Scott *et al.*, 2004) have established that microbial activity is evident, but no time-kill studies have been undertaken with *A. afra*. This report serves to validate the antimicrobial activity more quantitatively and investigate further, the role of the major compounds as possible antimicrobial constituents, thus providing a scientific basis for the traditional uses of *A. afra*.

4.2 Botanical description

Artemisia is a large and widespread genus in the family of Asteraceae housing approximately 350 species (Tan *et al.*, 1998). Most of the *Artemisia* species yield essential oils (Pappas and Sturtz, 2001). There is one indigenous (*A. afra*) and one localized (*A. vulgaris*) *Artemisia* species in Southern Africa. *Artemisia afra* is commonly known as African wormwood, "umhlonyane" (Xhosa, Zulu), "lengana" (Sotho, Tswana) and "wildeals" (Afrikaans) (van Wyk *et al*., 1997). This plant is a multi-stemmed perennial shrub, growing up to two meters in height. Glaucous feather-like leaves (Figure 4.1) with a pungent aromatic smell, indicative of its rich essential oil, characterize *A. afra*. The oil is yellow-green. The flowers are yellow, inconspicuous and borne at the ends of branches (von Koenen, 2001).



Figure 4.1 The leaves of *Artemisia afra*.

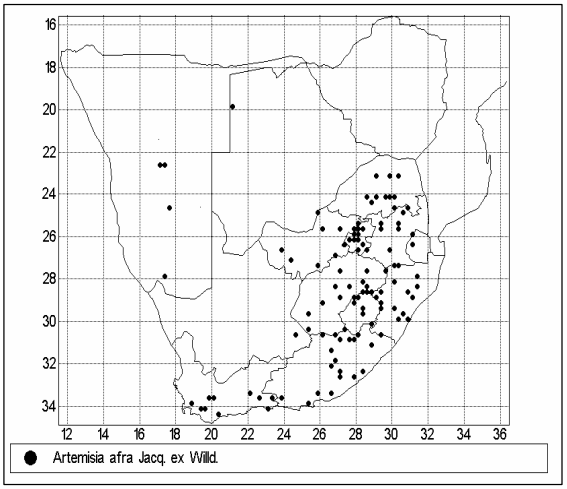


Figure 4.2 The geographical distribution of *Artemisia afra* in southern Africa (SANBI).

4.3 Distribution

The plant is abundantly distributed in mountainous regions from South Western Cape, extending from the Cederberg Mountains northwards. Distribution generally proceeds along the eastern coast through to the Northern Province (van Wyk *et al*., 1997). Figure 4.2 illustrates the geographical distribution of *A. afra* in South Africa.

4.4 Medicinal uses

Members of the genus have been frequently used to treat infections caused by bacteria, fungi and viruses. Watt and Breyer-Brandwijk (1962) reports the inhalation of leaf deconcoctions for blocked nose, headache, or alternatively the tip of a fresh plant is inserted into the nose to treat the same conditions. Fresh twigs may also be inserted into a hollow tooth to relieve toothache. The plant is usually burned and used as an inhalant for ailments of the bronchial passages. This suggests the release of volatile components and hence this study is narrowly focused on the essential oils of this species. The oils of the plant impart a bitter taste and the presence of thujone makes this plant toxic if consumed in sufficient quantities (Hutchings *et al*., 1996). *Artemisia afra* has also been used for the treatment of ailments associated with the gastrointestinal tract, fever and topical inflammation (von Koenen, 2001; Scott *et al*., 2004). In addition, there have been reports where *A. afra* has been combined with *Agrimonia bracteata, O. asteriscoides* (Chapter 3), *Lippia javanica* (Chapter 5), and *Tetradenia riparia* to treat respiratory disorders (Hutchings *et al*., 1996).

4.5 Methods

4.5.1 Chemical aspects

Plant collection and distillation of essential oils: The aerial parts from a natural population were collected during the growing period (February) from Klipriviersberg, southern region of Gauteng. Due to the larger volumes of essential oil required for the antimicrobial assays, a collective sample from Klipriviersberg was used to obtain the essential oil. A voucher specimen (Table 4.1) is deposited in the Department of Pharmacy and Pharmacology, University of Witwatersrand. The plants were distilled as described in Chapter 2.5.1.

Table 4.1 Plant collection data for *A. afra*.

Plant	Voucher	Material distilled (g)	Essential oil yield (% w/w)
A. afra	SVV173	623.76	0.2

Gas chromatography combined with mass spectrometry (GC-MS): Oil samples were analyzed with GC-MS using the Hewlett-Packard 1800A GCD system operating under the same conditions as described in Chapter 2.5.1.

4.5.2 Antimicrobial aspects

Culture, media preparation and assays were undertaken according to the NCCLS (2003) guidelines and methods described by Carson *et al*. (1995), and Eloff (1998a), details of which can be referred to in Chapter 2.5.2. Three different antimicrobial assays (disc diffusion, minimum inhibitory concentrations and time-kill studies) were performed on the hydrodistilled oil. Four additional test micro-organisms were added to the study i.e. *Candida tropicalis*, *Klebsiella pneumoniae*, *Bacillus cereus* and a different strain of *S. aureus. Candida* spp. are prevalent not only in the urinary tract but are also a cause of lung abscesses in immunocompromised patients. *Klebsiella pneumoniae* has been associated with septicaemia, cystitis, urinary tract infections, wound infections and pneumonia with pyrogenic respiratory infections being one of the most prevalent causes of lung infections in hospitals (Bannister *et al.*, 2000). These organisms are often hospital acquired complicating antibiotic treatment. The use of *A. afra* to treat both respiratory infections and disorders of the gut (*B. cereus* being one of the possible causative organisms responsible for enteric food poisoning) may be attributed to one of these organisms and thus their inclusion in the study was warranted. The acceptable MIC control ranges for these additional test pathogens as determined within the Department of Pharmacy and Pharmacology, University of Witwatersrand is given in Table 4.2.

Table 4.2 Acceptable MIC ranges for controls (ciprofloxacin for bacteria and amphotericin B for the yeast.

Test organism	MIC controls (μg/mL)
Klebsiella pneumoniae (NCTC 9633)	0.12-1.30
Staphylococcus aureus (ATCC 12600)	0.12-1.00
Bacillus cereus (ATCC 11778)	0.20-1.00
Candida tropicalis (clinical strain)	1.25-2.50

Time-kill assays were employed according to methods as described in Chapter 2.5.2 investigating two pathogens (*K. pneumoniae* and *C. neoformans*). Oil concentrations of 0.063, 0.125, 0.25, 0.5 and 0.75% (0.6, 1.8, 3.0, 5.2 and 8.1 mg/mL respectively) were selected to determine death kinetics of *K. pneumoniae,* and oil concentrations 0.25, 0.5, 0.75, and 1% (3.0, 5.2, 8.1 and 9.5 mg/mL respectively) were selected to determine death kinetics of *C. neoformans.* Selection was based on preliminary assays to determine optimal death kinetic response. Results were done in duplicate and the logarithmic mean plotted in Figures 4.4-4.6.

Major compound investigation: Individual compounds, obtained from Sigma-Aldrich were 1,8-cineole, at 98.0% purity (Lot 1054365), artemisia ketone at 97.0% purity (Lot 344166/114801), α-thujone at 96.0% purity (Lot 014071/423103488) and a mixture of α & β-thujone composed of 70% α-thujone and 10% β-thujone (Lot 414439/112701). Investigation of the role of the four major compounds on the overall microbial activity was investigated by MIC determination and time-kill analysis. The MIC assay was determined for all four major compounds against ten pathogens to determine if the individual compounds contribute towards the overall antimicrobial activity of *A. afra.* Assays were done in triplicate and the mean documented in Table 4.5. Individual compounds (Sigma-Aldrich) were prepared at starting concentrations 128 mg/mL and assayed with *A. afra.* After the addition of INT, results were observed after six hours (for bacteria) and 24 hr (for yeasts), as per standard MIC methodology described in Chapter 2.5.2. The results were left for a further 24 hr and re-examined to observe if any change to the original results occurred i.e. any loss of bacteriostatic action or regrowth. To examine whether any of these compounds independently or in combination (1,8-cineole and artemisia ketone; α & β-thujone; α-thujone and 1,8-cineole; α & β-thujone and 1,8-cineole; artemisia ketone and α-thujone; artemisia ketone and α & β-thujone; artemisia ketone, α-thujone and 1,8-cineole as well as artemisia ketone, α & β-thujone and 1,8-cineole) were responsible for bactericidal efficacy in *A. afra*, each compound and various combinations thereof were studied by death kinetic assay at the original concentration to that found in the plant (1,8-cineole at 17.8%, artemisia ketone at 10.1%, α-thujone at 18.8% and β-thujone at 12.5%). The number of colony forming units were determined at 0 min, 15 min, 240 min, 8 hr and 24 hr when subjected to *K. pneumoniae.* Time-kill assays were done in duplicate and the mean CFU counts documented in Table 4.6.

4.6 Results and discussion

4.6.1 Essential oil chemistry

Forty nine compounds were identified in the essential oil of *A. afra* representing 99.7% of the total composition (Table 4.3). Previous reports (Graven *et al.*, 1992**;** van Wyk *et al.*, 1997; Chagonda *et al.*, 1999) have shown that the major components of *A. afra* essential oils from different localities exhibited chemical variation. For instance, the major component reported from Ethiopian oils was artemisyl acetate (24.4 -32.1%), 1,8-cineole (63.4%) in the Kenyan oil, α and β-thujone (52.0%) in the Zimbabwean oil and α-thujone (52.5-54.2%) in the South African oil. In a more recent study (Viljoen *et al.*, 2006) the aerial parts of sixteen plants from four natural populations within South Africa were hydrodistilled and the essential oil analyzed by GC-MS. *Artemisia afra* displayed chemical variation in both the major and minor essential oil compounds within and between natural populations. Even though the Klipriviersberg sample was selected for this antimicrobial study one must be cognizant of the chemical variation that exists and take into account the possible variability that might arise should other populations or other individual plants be comparatively investigated.

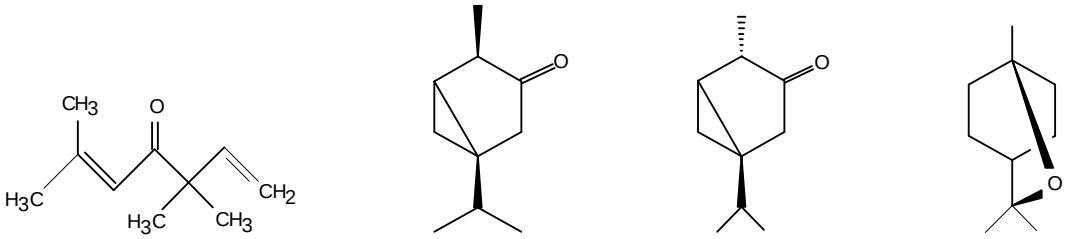
Table 4.3 Essential oil composition of *A. afra*.

RRI*	Compound name	Area percentage
1032	α-pinene	0.2
1043	santolinatriene	0.1
1076	camphene	1.0
1118	ß-pinene	0.1
1132	sabinene	1.0
1174	myrcene	0.1
1195	dehydro-1,8-cineole	tr
1213	**1,8-cineole**	**17.8**
1280	*p*-cymene	2.2
1358	**artemisia ketone**	**10.1**
1403	yomogi alcohol	4.9
1405	santolina alcohol	2.0
1409	santolinyl acetate	0.2
1437	**α-thujone**	**18.8**
1448	artemisyl acetate	1.8
1451	**ß-thujone**	**12.5**
1474	*trans*-sabinene hydrate	0.3
1497	α-copaene	0.1
1510	artemisia alcohol	5.5

RRI*	Compound name	Area percentage
1532	camphor	8.2
1540	chrysanthenone	0.4
1556	*cis*-sabinene hydrate	0.3
1571	*trans*-*p*-menth-2-en-1-ol	1.0
1586	pinocarvone	0.2
1597	bornyl acetate	0.2
1611	terpinen-4-ol	2.1
1638	*cis*-*p*-menth-2-en-1-ol	0.7
1643	dehydrosabina ketone	0.1
1651	sabinaketone	tr
1658	sabinyl acetate	0.1
1664	*trans*-pinocarveol	0.1
1682	δ-terpineol	0.1
1689	*trans*-piperitol	0.5
1706	α-terpineol	0.2
1719	borneol	2.7
1742	ß-selinene	0.2
1748	piperitone	0.7
1758	*cis*-piperitol	0.8
1786	*ar*-curcumene	0.1
1802	cumin aldehyde	0.1
1864	*p*-cymen-8-ol	0.1
1889	ascaridol	0.3
1957	*epi*-cubebol	0.1
2008	caryophyllene oxide	0.3
2113	cumin alcohol	0.1
2126	4-hydroxy-4-methylcyclohex-2-enone	0.1
2144	spathulenol	0.5
2209	T-muurolol	0.5
2264	intermedeol	0.2
	TOTAL	**99.7**

* RRI: Relative retention indices calculated against *n*-alkanes
% calculated from TIC data
tr = trace (< 0.1%)

Major compounds: The four major compounds (Figure 4.3) in the essential oil of *A. afra* are 1,8-cineole (17.8%), artemisia ketone (10.1%), α-thujone at 18.8% and β-thujone at 12.5% representing 59.2 % accumulatively.



artemisia ketone α-thujone β-thujone 1,8-cineole

Figure 4.3 Chemical structures for major compounds identified in the essential oil of *A. afra*.

4.6.2 Antimicrobial activity

Qualitative disc diffusion and quantitative minimum inhibition concentration (MIC) methods were used to evaluate antimicrobial activity. From the disc diffusion and MIC results presented in Table 4.4, it is clear that some antimicrobial activity is evident for *A. afra*. This confirms findings by Mangena and Muyima (1999) who reported the activity of African wormwood oil towards *B. subtilis, E. coli, K. pneumoniae* and *S. aureus.* According to Graven *et al*. (1992), the oil has value as a biological agent with greater antimycotic than antibacterial activity. Both author's results and that reported here, indicate *K. pneumoniae* as the most sensitive bacterial test organism in the disc diffusion assay.

Further correlations were difficult as collection locality, methodology, reference culture strains and interpretation of results differed. While Graven *et al.* (1992) reported antimicrobial activity against three mould species, in this study no fungal activity was found when assayed by disc diffusion against *A. alternata* and *A. niger*. As the assay used to determine antifungal activity differed it may account for the variation. Methodology variation is discussed in greater detail in Chapter 11. Graven *et al*. (1992) also made no microbial evaluation on the yeast species. In this study, the yeasts displayed some activity with *C. tropicalis* indicating the highest sensitivity (5 mm) for the disc diffusion assay. Gundidza *et al*. (1993), using the same methodology as Graven *et al.* (1992), has shown significant antifungal activity against *C. albicans, A. alternata* and *A. niger*.

The different localities from which the species were collected can differ in the essential oil composition and this may impact on the microbiological activities being reported. On examination of the literature, it can be noted that *A. afra* collected from Fort Hare (in the Eastern

Cape) had higher α-thujone (78.7%), β-thujone (13.1%) and lower 1,8-cineole (8.2%) content than that reported by Graven *et al*. (1992) and the oil did not contain any camphor (Mangena and Muyima, 1999). An antimicrobial and geographical variation study indicated that the antimicrobial activity within plants from a single population and inter-population variation was evident (Viljoen *et al.*, 2006). Such variation could account for discrepancies observed when comparatively assessing results from various researchers.

The MIC values for the essential oil from *A. afra* ranged between 8-16 mg/mL for most pathogens. The organism *S. odorifera* showed slightly higher sensitivity (4 mg/mL) and *S. typhimurium* showed lower sensitivity (32 mg/mL). The yeasts *C. neoformans, C. tropicalis* and *C. albicans* showed consistent (8 mg/mL) antifungal activity.

Table 4.4 Disc diffusion (mm from edge of disc) and MIC (mg/mL) of *A. afra* essential oil.

Test organism	**Disc diffusion (mm) [*1]**	**Disc diffusion controls[*2]**	**MIC (mg/mL)**	**MIC controls[*4]**
Pseudomonas aeruginosa ATCC 9027	0	6	16	0.8×10^{-3}
Klebsiella pneumoniae NCTC 9633	3	6	16	0.8×10^{-3}
Escherichia coli ATCC 11775	2	7	16	0.04×10^{-3}
Salmonella typhimurium ATCC 14028	2	5	32	0.8×10^{-3}
Serratia odorifera ATCC 33132	<1	4	4	0.4×10^{-3}
Staphylococcus aureus ATCC 12600	2	7	8	0.5×10^{-3}
Staphylococcus epidermidis ATCC 2223	1	12	16	0.4×10^{-3}
Enterococcus faecalis ATCC 29212	1	5	8	2.0×10^{-3}
Bacillus cereus ATCC 11778	1	10	16	0.8×10^{-3}
Bacillus subtilis ATCC 6051	3	8	16	0.1×10^{-3}
Cryptococcus neoformans ATCC 90112	3	8	8	2.5×10^{-3}
Candida tropicalis (clinical strain)	5	8	8	2.5×10^{-3}

Test organism	Disc diffusion (mm) [*1]	Disc diffusion controls[*2]	MIC (mg/mL)	MIC controls[*4]
Candida albicans ATCC 10231	1	8	8	1.3×10^{-3}
Aspergillus niger (clinical strain)	0	5	ND[*3]	ND[*3]
Alternaria alternata (clinical strain)	0	4	ND[*3]	ND[*3]

[*1] The zone of inhibition is expressed in mm from the disc edge.
[*2] Neomycin and nystatin served as controls for bacteria and fungi respectively.
[*3] ND = Methods did not incorporate MIC's for moulds.
[*4] Ciprofloxacin and amphotericin B served as controls for bacteria and yeasts respectively.

The pathogens *C. neoformans* and *K. pneumoniae* indicating moderate antimicrobial sensitivities in the disc diffusion assay were used to demonstrate the killing rate over time in the death kinetic studies. The time-kill plots for *K. pneumoniae* (Figure 4.4 and 4.5) demonstrate the concentration dependent antimicrobial activity where the bactericidal efficacy is greatest at 0.75% (8.1 mg/mL) within 10 min, followed by 0.5% (5.2 mg/mL) within 30 min, 0.25% (3.0 mg/mL) within 60 min and 0.125% (1.8 mg/mL) within 24 hr. Very little antimicrobial activity was noted at concentration 0.063% (0.6 mg/mL) and the growth curve was very similar to the control having no essential oil. As the time-kill response for *K. pneumoniae* is mostly within the first two hours, an enlarged section of Figure 4.4 is included to depict the detailed cidal activity occurring within the first hour (Figure 4.5). The positive control ciprofloxacin resulted in an immediate death response and due to presentation scale was not included in the time-kill plot. Time-kill plots for *C. neoformans* (Figure 4.6) do not follow a concentration dependent cidal pattern, however, all concentrations (0.25% to 1%) have a cidal effect within four hours. Concentrations 0.75% (8.1 mg/mL) and 1% (9.5 mg/mL) show similar death kinetic patterns, having bactericidal activity at 60 min. At a concentration of 0.25% (3.0 mg/mL), after an initial drop in CFU's over time, there was a levelling off of viability before bactericidal activity was reached at two hours. At a concentration of 0.5% (5.2 mg/mL) the *A. afra* essential oil followed a steady concentration dependent bactericidal tendency but after one hour the reduction of CFU's over time decreased at a much slower rate than that of the other higher concentrations.

The use of the essential oil of *A. afra* to treat respiratory ailments is validated by showing this rapid cidal rate over time for *K. pneumoniae* and *C. neoformans*, both respiratory pathogens associated with lung infections.

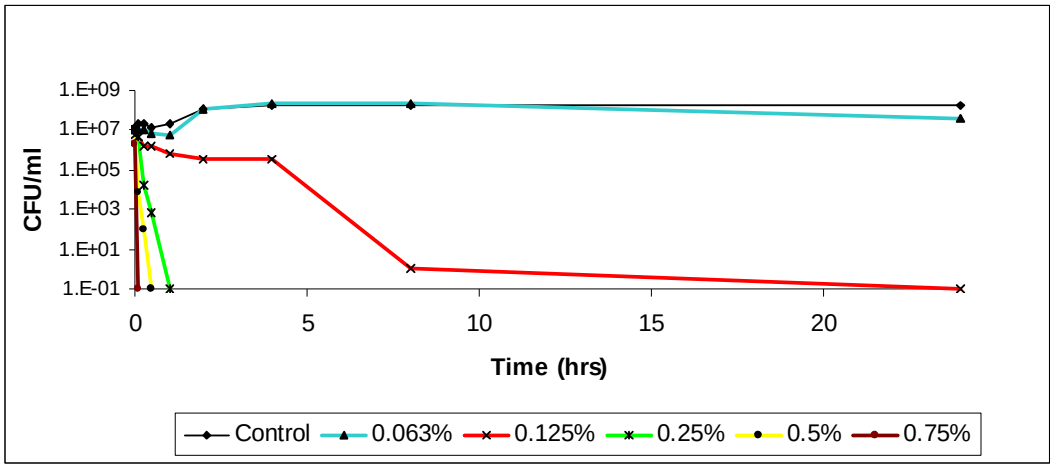


Figure 4.4 The death kinetics of *K. pneumoniae* on exposure to *A. afra* essential oil at concentrations 0.063%-0.75% over a 24 hr period.

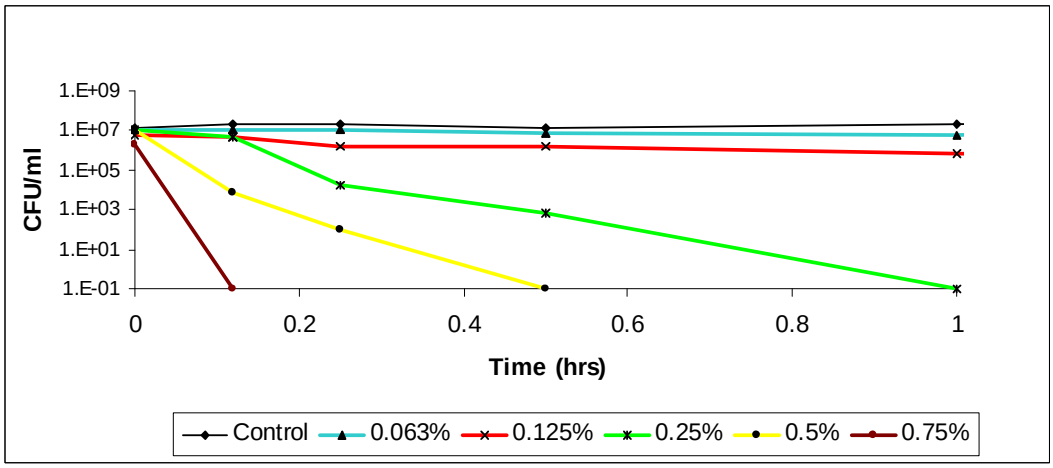


Figure 4.5 The death kinetic study of *K. pneumoniae* on exposure to *A. afra* essential oil showing bactericidal activity within one hour.

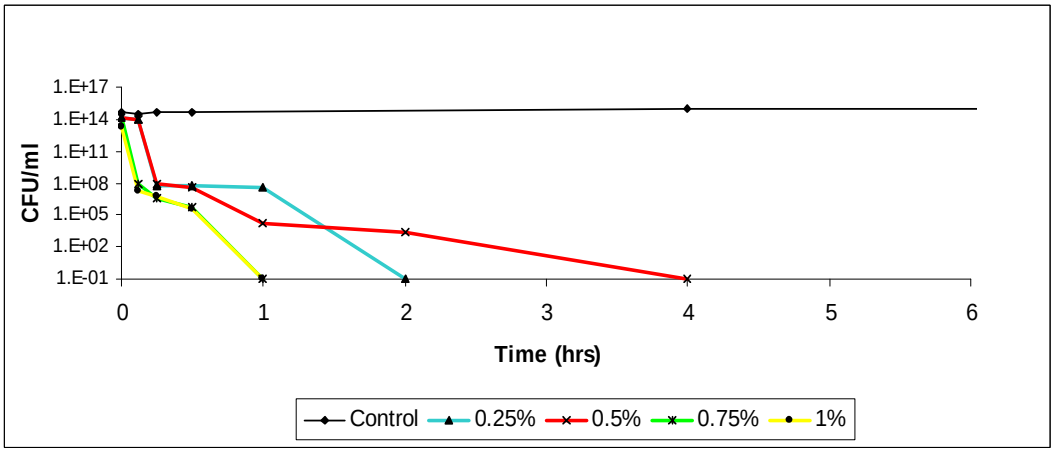


Figure 4.6 The death kinetics of *C. neoformans* on exposure to *A. afra* essential oil at concentrations 0.063%-0.75% over a six hour period.

The GC-MS results (Table 4.3) showed that the main chemical constituents in the essential oil of *A. afra* are α-thujone, β-thujone, artemisia ketone and 1,8-cineole. Previous antimicrobial studies on the constituent thujone have reported moderate to high activities (Dorman and Deans, 2000) and no activity (Hinou *et al.*, 1989) by means of disc diffusion assay. A more quantitative evaluation indicated poor activity by means of MIC determination (van Zyl *et al*., 2006). Thujone belongs to the aliphatic ketone group of constituents which is known for microbial inhibition rather than cidal efficacy (Pauli, 2001). Thujone is known for its toxicity (Kröner *et al.*, 2005; (http://www.sahealthinfo.org/tradionalmeds/monographs/artemisia.htm) and one would expect microbial cell destruction in response to the toxicity. However, thujone has very poor solubility (http://www.erowid.org/chemicals/absinthe_info3.shtml) and possibly this may account for the poor antimicrobial activities noted in this and previous studies.

For 10 of the 15 organisms previously studied (Table 4.4), the MIC of α-thujone, β-thujone, camphor, 1,8-cineole together with the essential oil from *A. afra* were recorded after six hours and 24 hr. The antimicrobial activity remained constant after the full 24 hr period for the essential oil of *A. afra*. The MIC values for almost all of the compounds against most of the pathogens studied increased indicating static rather than cidal activity for the independent compounds (Table 4.5). The regrowth after 24 hr suggests that other compounds are responsible for the cidal action noted for *A. afra*. A study undertaken by Setzer *et al.* (2004) where MIC's were determined for the major compounds (amongst others) in *A. douglasiana* also indicated that β-thujone, α-thujone, artemisia ketone and 1,8-cineole had no significant *in vitro* activity and it was thus suggested that further investigative procedures be carried out to determine which components are responsible for the activity found in *A. douglasiana*.

To further corroborate disc diffusion and MIC findings (Table 4.4) and take into account the possible synergistic role of the major compounds, time-kill studies were undertaken for various combinations of the major compounds: 1,8-cineole and artemisia ketone; α & β-thujone; α-thujone and 1,8-cineole; α & β-thujone and 1,8-cineole; artemisia ketone and α-thujone; artemisia ketone and α & β-thujone; artemisia ketone, α-thujone and 1,8-cineole as well as artemisia ketone, α & β-thujone and 1,8-cineole. Table 4.6, Figure 4.7 and Figure 4.8 illustrate the results (CFU/mL) obtained for the chemical constituents independently and in combination over a 24 hr time period. None of the compounds showed any bactericidal effect when investigated singularly over a 24 hour period with *K. pneumoniae*, thus corroborating findings

from the MIC studies. Of the eight combination of compounds, six (α & β-thujone; α-thujone and 1,8-cineole; artemisia ketone, α-thujone and 1,8-cineole; α & β-thujone and 1,8-cineole; artemisia ketone and α & β-thujone; artemisia ketone α- thujone and 1,8-cineole) showed a reduction in colony forming units after 15-240 min but regrowth after 24 hr. Compound combinations of artemisia ketone and 1,8-cineole as well artemisia ketone and α-thujone show no antimicrobial death kill over the 24 hr test period.

Table 4.5 MIC (mg/mL) determination for the major compounds and essential oil of *A. afra*.

Test organism	artemisia ketone		α-thujone		α and β thujone		1,8-cineole		*A. afra*		Control*
	6 hr	24 hr	6 hr	24 hr	6 hr	24 hr	6 hr	24 hr	6 hr	24 hr	
S. aureus ATCC 12600	16	>32	8	>32	16	>32	16	>32	8	8	1.0×10^{-3}
S. epidermidis ATCC 2223	16	16	16	>32	16	>32	16	>32	8	8	0.4×10^{-3}
B cereus ATCC 11778	8	32	8	>32	8	>32	8	>32	16	16	0.8×10^{-3}
B. subtilis ATCC 6051	16	32	16	32	8	32	16	>32	16	16	0.1×10^{-3}
E. coli ATCC 11775	16	>32	16	>32	16	>32	32	>32	16	16	0.04×10^{-3}
K. pneumoniae NCTC 9633	16	>32	8	>32	8	>32	16	>32	16	16	0.8×10^{-3}
S. typhimurium ATCC 14028	8	16	8	16	12	>32	32	>32	32	32	0.8×10^{-3}
S. odorifera ATCC 33132	16	16	16	>32	16	>32	16	>32	4	4	0.4×10^{-3}
C. albicans ATCC 10231	16	16	12	16	15	>32	16	>32	8	8	2.5×10^{-3}
C. neoformans ATCC 90112	32	32	>32	>32	6	>32	>32	>32	8	8	1.25×10^{-3}

* Ciprofloxacin and nystatin served as controls for bacteria and yeasts respectively.

Table 4.6 The CFU/agar plate obtained for the various major compound combinations together with *A. afra* essential oil tested against *K. pneumoniae*.

Chemical standard	Time Scale*[1]				
	0 min	15 min	240 min	8 hr	24 hr
*Klebsiella pneumoniae**[2]	>1000	>1000	>1000	>1000	>1000
0.5% *Artemisia afra*	>1000	208	0	0	0
1,8-cineole	>1000	>1000	>1000	>1000	>1000
artemisia ketone	>1000	>1000	>1000	>1000	>1000
α-thujone	>1000	>1000	>1000	>1000	>1000

Chemical standard	Time Scale*[1]				
	0 min	15 min	240 min	8 hr	24 hr
α and β-thujone	>1000	6	0	697	>1000
artemisia ketone + 1,8-cineole	>1000	>1000	>1000	>1000	>1000
α-thujone + 1,8-cineole	>1000	92	23	1003	>1000
α and β-thujone + 1,8-cineole	>1000	7	0	0	1023
artemisia ketone + α-thujone	>1000	>1000	>1000	>1000	>1000
artemisia ketone + α and β-thujone	>1000	90	1	19	>1000
artemisia ketone + α-thujone + 1,8-cineole	>1000	120	1	12	>1000
artemisia ketone + α and β-thujone + 1,8-cineole	>1000	15	0	0	>1000

*[1] Shaded areas indicate CFU >1000.
*[2] Control test organism without any compound or antimicrobial indicating acceptable growth kinetics.

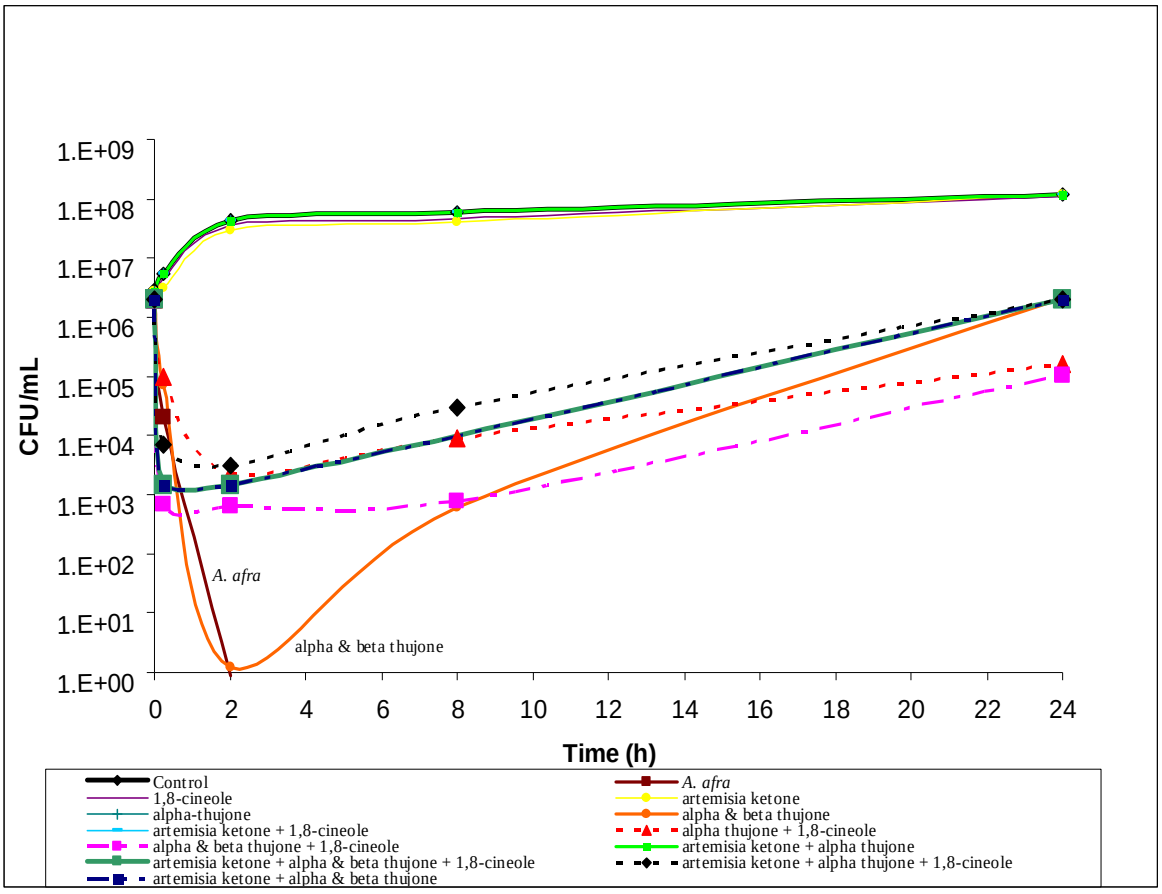


Figure 4.7 The death kinetic representation for the various major compound combinations together with *A. afra* (0.5%) essential oil tested against *K. pneumoniae*.

Chemical standard*	CFU at time intervals
	0 min 15 min 240 min 8 hr 24 hr
Klebsiella pneumoniae control	
0.5% *Artemisia afra*	
1,8-cineole	
α and β-thujone	
α-thujone + 1,8-cineole	
α and β-thujone + 1,8-cineole	
artemisia ketone + α-thujone + 1,8-cineole	
artemisia ketone + α & β-thujone + 1,8-cineole	

Figure 4.8 The CFU's observed when performing the compound combination time-kill assay tested against *K. pneumoniae.*

* Figure 4.8 represents only a selected portion of the study for illustrative purposes. Note that the study was done in duplicate, with a 5 times dilution made for all combinations and final mean CFU counts represented in Table 4.6 and Figure 4.7.

Both MIC and time-kill studies on the major compounds ascertain that the antimicrobial activities cannot be attributed to the major compounds or combination thereof. As suggested by Chalchat and Garry (1997), when investigating the correlation between chemical composition

and antimicrobial activity one needs to take into account less abundant components. The essential oil of *A. afra* has 49 compounds as determined by the GC-MS data. To determine the antimicrobial activity on each individual compound, as well as taking into consideration the possibility of synergy between compounds is not feasible as a certain number of trace compounds would still be unaccounted for. As seen in this study with *A. afra* essential oils, one or more of the 45 minor compounds are probably associated with the antimicrobial activity seen here.

The use of the whole *A. afra* plant to treat microbial infections is conclusively validated in this study. As noted, investigating single compounds to determine bioactivity does not always yield the complete pharmacological action and in searching for single bioactive compounds scientists may be overlooking other important synergistic interactions between constituents. Many phytomedicines on the market today, such as *Ginkgo biloba* and Kava kava, are sold as whole extracts and it is believed that the synergistic interactions between the constituents are responsible for their therapeutic efficacy (Williamson, 2001). It is also known that many traditional healers rely not only on a single plant constituent for therapeutic regimens but often combine various plant parts and even different species in the belief that efficacy may be enhanced. In this way the efficacy of *A. afra* may be biologically more active when all constituents within the plant are accounted for.

4.7 General conclusions

- The major constituents of the essential oil of *Artemisia afra* analysed in this study are 1,8-cineole at 17.8%, artemisia ketone at 10.1%, α-thujone at 18.8% and β-thujone at 12.5% representing 59.2 % accumulatively.

- The highest antimicrobial activities obtained by means of the disc diffusion assay were noted for the fungal organism *Candida tropicalis* (5 mm).

- The highest antimicrobial activity noted in the MIC assay was against *Serratia odorifera* (4 mg/mL).

- Rapid death kinetics for the respiratory pathogens *Klebsiella pneumoniae* and *Cryptococcus neoformans* verify the ethnobotanical use of *Artemisia afra* to treat respiratory infections.

- The major compounds of *Artemisia afra* when accessed independently and in combination had negligible antimicrobial activity, thus suggesting that the major chemical compounds may be acting in a synergistic manner with the minor compounds, or that the minor compounds, independently or in combination, may be responsible for the antimicrobial efficacy noted for *Artemisia afra*.

Chapter 5

Lippia javanica (Burm.f.) Spreng, the antimicrobial activity and an *in vitro* synergy study when combined with *Artemisia afra* to treat *K. pneumoniae* infections.

5.1 Introduction

The genus *Lippia* (Burm.f.) Spreng, a member of the Verbenaceae family is represented by approximately 200 herbs, shrubs and small trees which are often of an aromatic nature (Terblanché and Kornelius, 1996). *Lippia javanica* is commonly referred to as "fever tea". This name is probably derived from its use as a weak infusion for fever symptoms. In Afrikaans it is referred to as "koorsbossie", which literally translated means "fever bush". The Zulu name "Umsuzwane", refers to the strong odour of the plant (Ngwenya *et al*., 2003). The aromatic properties of the plant have resulted in its use both medicinally and for culinary purposes as a flavouring and a sweetener (Pascual *et al*., 2001). A number of species have been used in African tribal customs as a treatment to ward off demons and even in contradictory conditions such as a stimulant and sedative (Pascual *et al.*, 2001). The plant is used traditionally as a hand and body wash after burial procedures (Ngwenya *et al*., 2003). While a number of authors have reported on the chemistry of the genus (Velasco-Negueruela *et al*., 1993; Terblanché and Kornelius, 1996; Chagonda and Makanda, 2000; Braga *et al*., 2005), antimalarial properties (Valentin *et al.*, 1995) and the pharmacology (Pascual *et al.*, 2001), the species *L. javanica* has been neglected with regard to antimicrobial studies. Manenzhe *et al.*, (2004), has reported briefly on the antimicrobial activity. Viljoen *et al*. (2005), highlighted the chemical variation in natural populations in South Africa as well as the antimicrobial efficacy for *L. javanica* against three pathogens. The linalool chemotype has been investigated in this Chapter to further elaborate on the antimicrobial activities. The combination of *L. javanica* with *A. afra* as an antimicrobial is also studied. While literature sites the traditional use of plant combinations (Hutchings *et al*., 1996; van Wyk and Gericke, 2000) there has been very little scientific evidence to support the traditional use of *L. javanica* in combination with *A. afra*.

5.2 Botanical description

Lippia javanica is an erect woody shrub approximately two metres in height. Its hairy leaves have compound veins and are highly aromatic with a strong lemon smell. Dense rounded heads of small yellowish-white flowers (Figure 5.1) appear during the flowering season (van Wyk *et al.*, 1997).



Figure 5.1 The aerial leaves and white flower heads of *Lippia javanica.*

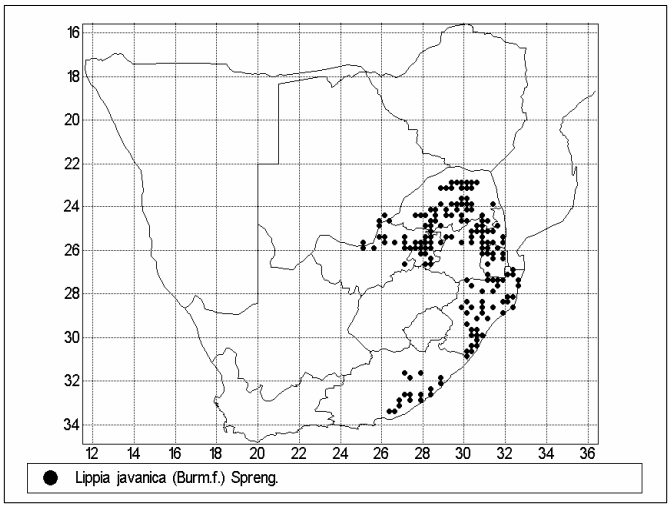


Figure 5.2 The geographical distribution of *L. javanica* in South Africa (SANBI).

5.3 Distribution

The plant is distributed in southern Africa and northwards, mainly in the eastern regions. Plant populations extend from the Eastern Cape through to KwaZulu-Natal, Mpumalanga, Gauteng, North-West Province and Northern Province with a few *L. javanica* populations present in southern Botswana (Figure 5.2).

5.4 Medicinal uses

Both the layman and traditional healers use the plant extensively in traditional medicine to treat minor ailments. The leaves and stems are often used and in some cases the roots as well (van Wyk *et al.*, 1997). Strong leaf infusions are made, which are commonly used as inhalants and

also topically for scabies and lice (van Wyk *et al*., 1997; Gelfand *et al*., 1985). In Tanzania, the species is used to treat skin infections where fresh bruised leaves are rubbed over the infected skin area (Ngassapa *et al*., 2003). More commonly, leaf and stem infusions are used as a tea to treat coughs, colds, fever and bronchitis (Watt and Breyer-Brandwijk, 1962; Hutchings *et al*., 1996). It has also been used for bronchial ailments and influenza (Hutchings *et al*., 1996). Roots are used as antidotes for suspected food poisoning and bronchitis. The Vhavenda people use leaf infusions as antihelmintics, respiratory and febrile ailments and as a prophylactic against dysentery, diarrhoea and malaria (Mabogo, 1990). Roots are used as antidotes for suspected food poisoning, for bronchitis and sore eyes (Hutchings *et al*., 1996). Ethnobotanical literature documents its uses for fever and influenza in combination with leaves of *A. afra* (Hutchings *et al*., 1996).

5.5 Methods

5.5.1 *Chemical aspects*

Plant collection and distillation of essential oils: The aerial parts of the plants were collected in the growing season (November) from a natural population in Fairland, in the northern regions of Gauteng. A voucher specimen (Table 5.1) is retained in the Department of Pharmacy and Pharmacology, University of Witwatersrand. The plant material was distilled, as described in Chapter 2.5.1.

Table 5.1 Plant collection data for *L. javanica*.

Plant	Voucher	Material distilled (g)	Essential oil yield (% w/w)
L. javanica	AMV355	6517.9	0.2

Gas chromatography combined with mass spectrometry (GC-MS): Oil samples were quantitatively analyzed with GC-MS using the Hewlett-Packard 1800A GCD system operating under the same conditions as described in Chapter 2.5.1.

5.5.2 Antimicrobial aspects

Culture, media preparation and assays were undertaken according to the NCCLS (2003) guidelines and methods described by Carson *et al*. (1995) and Eloff (1998a) in Chapter 2.5.2. Three different antimicrobial assays (disc diffusion, MIC and time-kill studies) were performed on the hydrodistilled oil. For the time-kill assay oil concentrations of 0.25, 0.5, 0.75 and 1% (3.0, 5.2, 8.1 and 9.5 mg/mL respectively) were selected to determine the death kinetics of *K. pneumoniae, C. neoformans* and *B. cereus.* The MIC was determined on a racemic mixture of linalool at 95% purity (Lot 409782, Sigma-Aldrich) at a starting concentration of 128 mg/mL, against all bacterial and yeast isolates from this study. Time-kill assays were employed according to methods as described in Chapter 2.5.2 investigating three pathogens (*K. pneumoniae, B. cereus* and *C. neoformans*). Results were recorded in duplicate and the mean plotted in Figures 5.4-5.6.

A time-kill assay was used to assess the ethnobotanical use of *L. javanica* with *A. afra* in combination therapy against the respiratory pathogen *K. pneumoniae.* Essential oil at 0.25% concentration obtained from *L. javanica* and *A. afra* (Chapter 4.5.1) were run independently with a combination of *L. javanica* and *A. afra* (together totalling 0.25%). A control (*K. pneumoniae* having no essential oil) was included in the study. Aliquots were sampled (as described in Chapter 2.5.2.) at pre-determined time intervals ranging from 0 min - 48 hr, (5 min, 15 min, 240 min, 8 hr, 24 hr and an extended 48 hr). Colony-forming units were counted and death kinetics expressed in Table 5.4 and Figure 5.7. The study was done in duplicate.

5.6 Results and discussion

5.6.1 Essential oil chemistry

Thirty-four compounds were identified in the essential oil of *L. javanica* representing 99.40 % of the total composition (Table 5.2).

Table 5.2 Essential oil composition of *L. javanica.*

RRI*	Compound name	%
1032	α-pinene	0.2
1076	camphene	0.3
1174	myrcene and α-phellandrene	2.6

RRI*	Compound name	%
1203	limonene	0.5
1218	β-phellandrene	0.5
1246	**(*Z*)-β-ocimene**	**13.0**
1266	(*E*)-β-ocimene	6.2
1280	p-cymene	0.9
1319	dihydrotagetone	0.2
1382	*cis*-alloocimene	0.2
1444	ipsenone (tentative, Wiley)	0.8
1450	*trans*-linalool oxide (*furanoid*)	0.2
1452	1-octen-3-ol	0.1
1478	*cis*-linalool oxide (*furanoid*)	0.2
1497	α-copaene	0.4
1500	*cis*-tagetone	0.2
1522	*trans*-tagetone	0.3
1532	camphor	0.2
1553	**linalool**	**65.2**
1612	β-caryophylline	3.6
1661	alloaromadendrene	0.1
1687	α-humulene	0.2
1704	γ-muurolene	0.1
1719	borneol	0.4
1726	germacrene-d	1.5
1740	α-muurolene	0.1
1755	bicyclogermacrene	0.1
1758	(*E*,*E*)-α-farnesene	0.2
1773	δ-cadinene	0.2
1830	2,6-dimethyl-3(*E*),5(*E*),7-octatriene-2-ol	0.1
2001	isocaryophylline oxide	0.1
2008	caryophylline oxide	0.4
2050	(*E*)-nerolidol	0.1
2071	humulene epoxide II	tr
	TOTAL	**99.40**

*RRI: Relative retention indices calculated against n-alkanes
% calculated from TIC data
tr = trace <0.1%.

Major compounds: The three major compounds (Figure 5.3) in the essential oil of *L. javanica* are linalool (65.2%) and (*Z*)-β-ocimene (13.0%), representing 78.2% accumulatively.



linalool (*Z*)-β-ocimene

Figure 5.3 Chemical structures for the major compounds identified in the essential oil of *L. javanica*.

Mwangi *et al*. (1992) and Terblanché and Kornelius (1996) have cited myrcene, myrcenone, ocimene, (*E*)-tagetenone, (*Z*)-tagetenone and *cis*-tagetone as major compounds in *L. javanica.* Of these, only (*Z*)-β-ocimene is present as a major constituent in this sample analysed from the Fairland population. Variation in chemical composition was also noted by Ngassapa *et al*. (2003), when evaluating two different *L. javanica* samples from the same locality in Tanzania. Further analysis of the composition variation was undertaken (Viljoen *et al*., 2005) where sixteen samples (representing five natural populations) were investigated by cluster analysis and five chemotypes were identified: a myrcenone rich type (36–62%); a carvone rich type (61–73%); a piperitenone rich type (32–48%); an ipsenone rich type (42–61%) and a linalool rich type (>65%). The myrcenone and linalool chemotypes have been mentioned in literature (Chagonda and Makanda, 2000; Ngassapa *et al*., 2003; Terblanché and Kornelius, 1996) but the carvone, ipsenone and piperitenone chemotypes have not previously been reported for *L. javanica*. Of the five localities studied, the Fairland population was selected for further antimicrobial investigation. Selection was based on its chemotype (rich in linalool), not identified in any of the other samples, preliminary antimicrobial analysis (Subramoney, 2003) and the added advantage that the vast quantity of plant material required for time-kill analysis was easily accessible.

As linalool represents the major component of the essential oil, it warranted further assessment to determine if the antimicrobial efficacy may be attributed to this major constituent. The MIC (Table 5.3) was determined for a racemic mixture of linalool against nine reference bacterial strains and three yeast strains. Pattnaik *et al*. (1997) reported moderate antibacterial sensitivity for linalool against *S. aureus, E. coli* and *K. pneumoniae*. For *C. albicans* and *C. neoformans* high sensitivities were found. Resistance was reported for *P. aeruginosa*. In this study only the bacterial results show partial congruency. Pattnaik *et al*. (1997) noted that MIC's from essential oils were in many cases lower than the major constituents independently suggesting that synergy

between constituents may be contributing to the enhanced activity. Minimum inhibitory concentration values for *L. javanica* essential oil indicated higher sensitivities than the linalool constituent for pathogens *P. aeruginosa, C. albicans* and *C. neoformans.* In another study (Inouye *et al*., 2001a) where the vapour was analysed for antimicrobial activity, linalool was assessed with 14 other essential oils. Results for an oil rich in linalool (Coriander, 73.2% linalool) indicted the same MIC value for linalool and Coriander against *S. aureus* and *E. coli.* These organisms together with *C. tropicalis* also gave linalool MIC results equivalent to that found in the *L. javanica* essential oil signifying that linalool may be responsible for the antimicrobial activity noted against these pathogens. In a more recent study (Alviano *et al*., 2005), indicated by means of a bio-autographic assay, that the linalool rich essential oil of *Croton cajucara,* while indicating antimicrobial activity, showed no antibacterial effect against the separated linalool constituent. While the study by Alviano *et al*. (2005) seems to contrast with previous results obtained by Inouye *et al*. (2001a), one must take into account that different pathogens were used to assess antimicrobial activity. The study by Alviano *et al*. (2005) was restricted to oral bacterial biofilms only. As noted earlier, the MIC's obtained for linalool in this study with *L. javanica* also indicated no activity with selected pathogens. Thus, the antimicrobial activity may or may not be attributed to the major constituent linalool (65.2%), depending on the pathogen studied.

5.6.2 Antimicrobial activity

Antimicrobial disc diffusion and MIC assays (Table 5.3) were performed on the essential oil samples obtained from *L. javanica* and tested against five Gram-positive bacteria, four Gram-negative bacteria and five fungi. Highest disc diffusion sensitivities were noted for *K. pneumoniae* (5 mm), *E. faecalis* (4 mm) and *C. neoformans* (4 mm). Interestingly, these pathogens are associated with respiratory infections and hence the correlation to the traditional use of *L. javanica*. None of the oil samples showed any apparent activity in the disc diffusion assay against *P. aeruginosa, S. epidermidis* and the mould *A. niger*.

The MIC data has indicated moderate activity (8 mg/mL) for most of the bacterial isolates. No significant antimicrobial activity was found in the MIC assay for *L. javanica*. Ngassapa *et al*. (2003) indicated similar MIC results on one of the *L. javanica* species (sample B) collected from Tanzania, even though the chemical composition varied considerably to the chemical composition of the *L. javanica* sample collected from Fairlands. The authors documented

limonene (11.3%), neral (13.7%) and geraniol (21.5%) as major components whereas linalool (1.8%) and β-ocimene (*trans* 0.3% and *cis* 0.3%) were only present as minor components. Previous reports (Knobloch *et al*., 1989) on ocimene have shown poor to moderate antimicrobial activity and no conclusive correlation could be made with the major compound ocimene and the antimicrobial activity noted in *L. javanica*.

Table 5.3 Disc diffusion (mm from edge of disc) and MIC (mg/mL) of *L. javanica* essential oil.

Test organism	**Diameter (mm) [*1]**	**DD controls[*2]**	**MIC (mg/mL)**	**Linalool**	**MIC controls[*4]**
P. aeruginosa ATCC 9027	0	6	8	16	1.00×10^{-3}
K. pneumoniae NCTC 9633	5	5	16	4	0.13×10^{-3}
E. coli ATCC 11775	1	5	4	4	0.08×10^{-3}
S. odorifera ATCC 33132	1	6	8	4	1.25×10^{-3}
S. aureus ATCC 25923	2	10	16	16	0.50×10^{-3}
S. epidermidis ATCC 2223	0	12	8	4	0.63×10^{-3}
E. faecalis ATCC 29212	4	3	16	2	2.00×10^{-3}
B. cereus ATCC 11778	1	10	8	8	0.63×10^{-3}
B. subtilis ATCC 6051	1	10	16	8	0.09×10^{-3}
C. neoformans ATCC 90112	4	5	4	8	2.50×10^{-3}
C. tropicalis (clinical strain)	3	5	32	32	2.50×10^{-3}
C. albicans ATCC 10231	1.5	7	16	32	1.30×10^{-3}
A. niger (clinical strain)	0	7	ND[*3]	ND[*3]	ND[*3]
A. alternata (clinical strain)	0.5	3	ND[*3]	ND[*3]	ND[*3]

[*1] The diameter of the zone of inhibition is expressed in mm from the disc edge.
[*2] Neomycin and nystatin served as controls for bacteria and fungi respectively.
[*3] ND = Methods did not incorporate MIC's for moulds.
[*4] Ciprofloxacin and amphotericin B served as controls for bacteria and yeasts respectively.

There was very little correlation between disc diffusion and MIC data. For instance with *P. aeruginosa* and *S. epidermidis*, no antimicrobial activity was found in the disc diffusion assay, yet moderate activity (8 mg/mL) was noted in the MIC assay. Conversely, significant activity was noted for *K. pneumoniae* (5 mm) in the disc diffusion assay but lower sensitivities (16 mg/mL) noted in the MIC assay. Some correlation did exist, as in the case of *S. aureus* (2 mm; 16 mg/mL), *B. subtilis* (1 mm; 16 mg/mL), *C. neoformans* (4 mm; 4 mg/mL) and *C. albicans* (1.5 mm; 16 mg/mL). Method variation is further discussed in Chapter 11.

As *L. javanica* is mainly used in African traditional medicine to treat respiratory disorders such as coughs, colds and bronchitis, time-kill assays were performed on the three respiratory pathogens from three different micro-organism groups, *K. pneumoniae* (Gram-negative), *C. neoformans* (yeast) and *B. cereus* (Gram-positive) which are commonly associated with opportunistic infections in immune-compromised patients. The essential oil displays mostly moderate antimicrobial activity against respiratory pathogens in the disc diffusion and MIC methodology. By further examination using death kinetics, a more detailed pharmacodynamic approach may be observed for the oils when exposed to respiratory pathogens. This antimicrobial assay shows the bactericidal effect of the oil at concentrations 0.25, 05, 0.75 and 1%.

For *K. pneumoniae* (Figure 5.4), a total rapid bactericidal efficacy was reached within 30 min for all oil concentrations. The lower oil concentrations (0.25% and 0.5%) indicated an initial gradual decline in CFU within 20 min after which the viable bacterial counts were reduced rapidly. The higher oil concentration (0.75%) also indicated an initial rapid reduction in viability within 8 min and thereafter a gradual decline in viability. The highest oil concentration investigated (1%) indicated an immediate reduction in CFU over time resulting in very few viable colonies still evident after 7 min. Total cidal activity was obtained at 30 min. This correlates with the high disc diffusion activities noted for this pathogen.

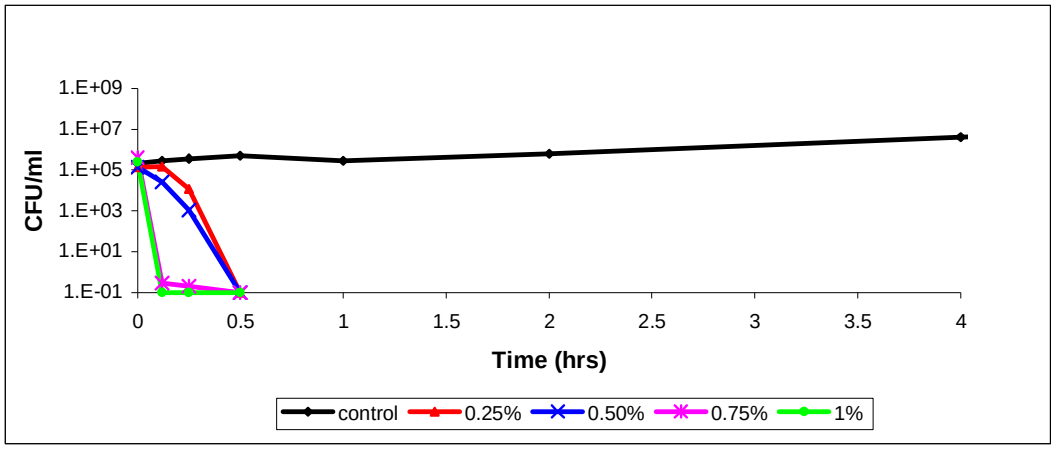


Figure 5.4 Time-kill plot of *L. javanica* essential oil showing death kinetics of *K. pneumoniae* (NCTC 9633) represented over the first four hours of a 24 hr test period.

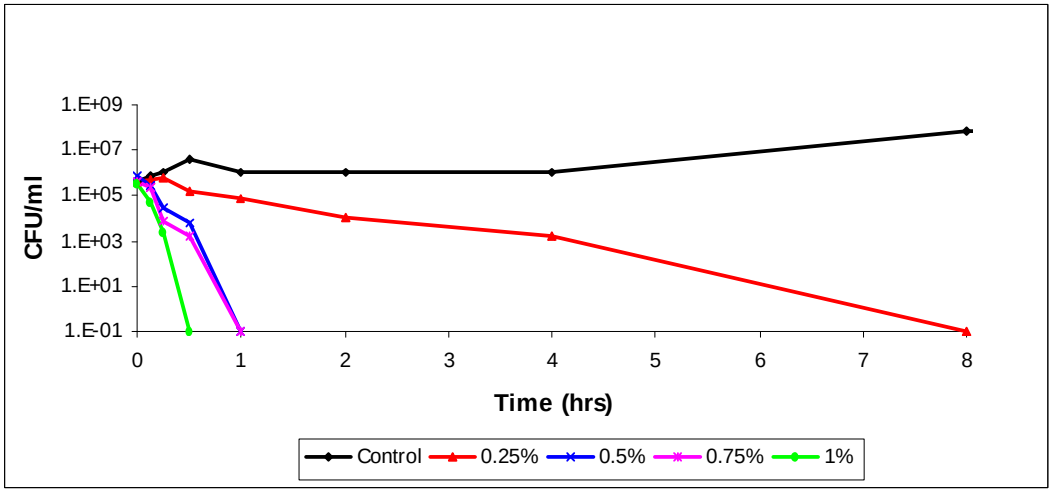


Figure 5.5 Time kill plot of *L. javanica* essential oil showing death kinetics of *C. neoformans* (ATCC 90112) represented over the first eight hours of a 24 hr test period.

Death kinetics for *C. neoformans* (Figure 5.5) showed a killing rate for concentrations 0.5, 0.75 and 1% within one hour. The lowest concentration of 0.25% (3 mg/mL) took eight hours before a cidal effect was noted and death kinetics correlate with the MIC value (4 mg/mL) obtained for this pathogen. While it initially appears that *L. javanica* is more effective against *K. pneumoniae*, one must take into account the growth response time of the organism *C. neoformans*. The growth response time is longer for yeasts, as seen in the control where growth only increases exponentially after four hours. Cidal efficacy is therefore expected to take longer. The MIC (4 mg/mL) and disc diffusion (4 mm) results indicate one of the highest sensitivities towards *C.*

neoformans and further substantiate the use of *L. javanica* essential oils to treat infections associated with this organism.

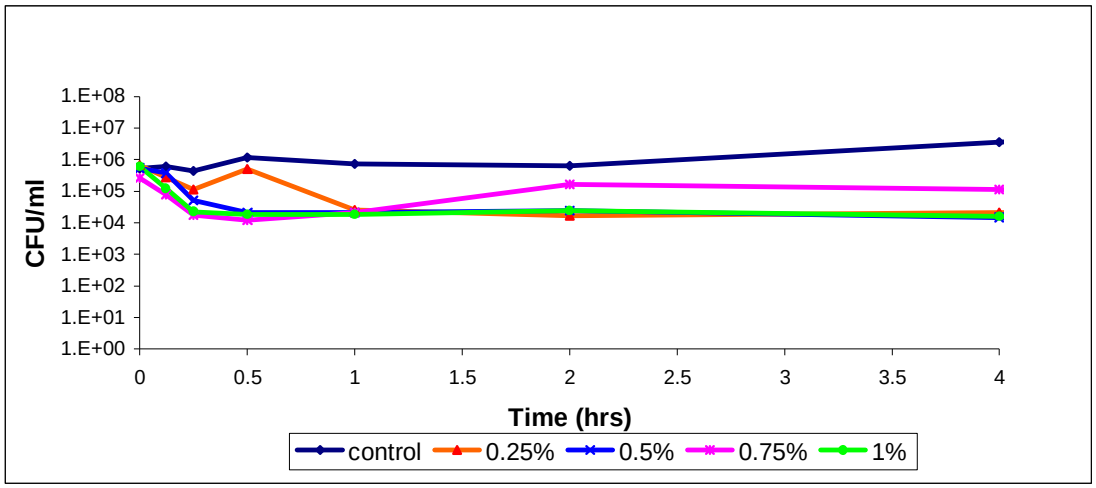


Figure 5.6 Time-kill plot of *L. javanica* essential oil showing death kinetics of *B. cereus* (ATCC 11778) represented over four hours of a 24 hr test period.

Bacillus cereus (Figure 5.6) showed a reduction in growth within 30 min for all the concentrations studied. Thereafter, growth maintained a generally consistent non-responsive pattern neither increasing nor decreasing CFU over the 24 hr test period. While this initially suggests that the essential oil of *L. javanica* has no significant effect on *B. cereus*, one must consider that the test pathogen does contain spores and while initial results show a reduction in CFU possibly suggesting the cidal efficacy against vegetative cells, the later growth could possibly be due to the germination of spores. When comparatively examining the growth response of the essential oil concentrations against the control after 30 min, it can be noted that the CFU remained consistently lower than the control. This indicates that a bacteriostatic effect is occurring. In therapeutic treatment regimens, a patient is usually given more than one antimicrobial treatment dose and one could postulate that if another dose (at the concentration given initially) of essential oil were to be introduced after 30 min (when cidal action begins to cease) it may have resulted in cidal action instead of the static effect noted. The death kinetic plot for *B. cereus* does correlate with the disc diffusion assay (1 mm) and to a lesser extent the MIC (8 mg/mL).

Comparison of the time-kill plots for the three organisms studied showed that the killing rate was the greatest for *K. pneumoniae*, thereafter *C. neoformans* and very little reduction of microbial populations for *B. cereus.*

A time-kill study was undertaken with *L javanica* and *A. afra*, two plants often used in combination therapy to treat respiratory infections amongst the ethnic populations. The combination time-kill study for *L. javanica* and *A. afra* (Figure 5.7) and tabulated results (Table 5.4) are shown for each plant independently and in combination. *Artemisia afra* when studied independently showed initial microbial destruction within one hour, but regrowth after 24 hr. These results correlated with the death kinetics performed on *A. afra* found in Figure 4.4 (Chapter 4) where cidal activity was seen within one hour, however, the extended assay given here over 48 hr indicated that the antimicrobial efficacy for *A. afra* at 0.25% could not be maintained by long term exposure as regrowth occurred after 24 hr. For the *L. javanica* oil at 0.25%, death kinetics was observed within 40 min but regrowth after four hours. As with *A. afra*, the regrowth was not noted in previous death kinetic assays on the same oils. The microbial load was increased in this study and this could account for the extra bioburden placed on the oils. When the two plants were combined, a bactericidal effect was maintained for the full 48 hours of testing. This synergistic effect scientifically validates the combined use of *L. javanica* and *A. afra* for the treatment of respiratory infections associated with *K. pneumoniae* and corroborates with the traditional use of these two plants when used in combination.

Table 5.4 The CFU on agar plate obtained for 0.25% essential oils against *K. pneumoniae* of *L. javanica* and *A. afra* independently and in combination.

Sample	**CFU over time***					
	0 min	15 min	1 hr	8 hr	24 hr	48 hr
K. pneumoniae control	>1000	>1000	>1000	>1000	>1000	>1000
Artemisia afra (0.25%)	>1000	300	0	0	0	>1000
Lippia javanica (0.25%)	>1000	>1000	0	1000	>1000	>1000
A. afra (0.125%) and *L. javanica* (0.125%)	>1000	>1000	0	0	0	0

***** Shaded areas indicate CFU >1000.

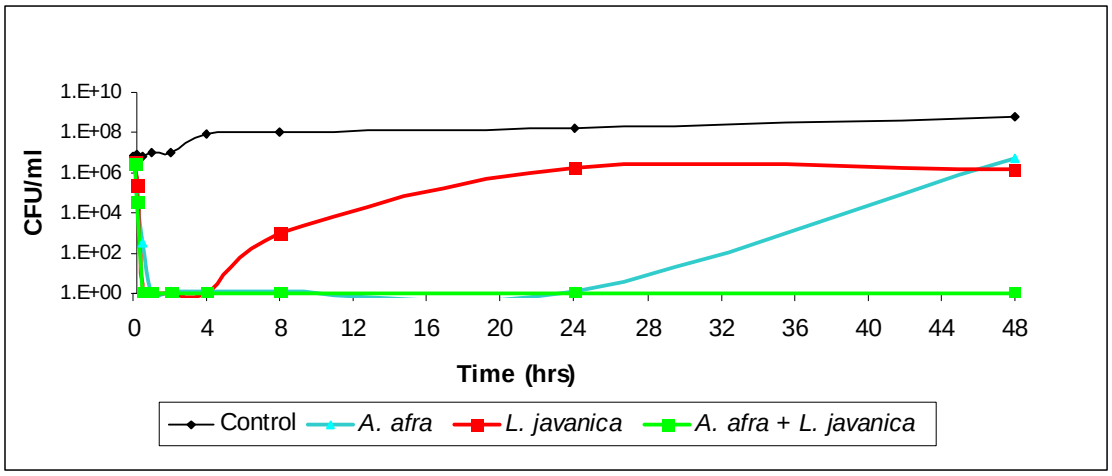


Figure 5.7 Time-kill plot of *L. javanica* and *A. afra* independently and in combination for 0.25% essential oil against *K. pneumoniae* (NCTC 9633) represented over 48 hr.

A review of the literature on plant combination therapy revealed that no death kinetic assays have been previously presented for plant combinations. A disc diffusion study was undertaken (Jain and Kar, 1971; Geda, 1995), where oils were mixed and applied to discs in a 1:1 ratio. These studies have shown selective synergy for some oils. In another study (Lachowicz *et al*., 1998) linalool was combined with methyl charvicol at v/v ratios of 1:0; 0.8:0.2; 0.6:0.4; 0.4:0.6; 0.2:0.8 and 0:1. It was observed that the combined concentrations showed higher efficacies than the constituents independently, when tested against two test micro-organisms. Furthermore, medicinal plants administered by traditional healers often make use of a combination of species to enhance efficacy. A comprehensive study on the ethnobotanical use of plant mixtures is given by Cano and Volpato (2004), where 170 plant species from Cuba were examined for their combined medicinal use. Other pharmacological studies on plant species have also been undertaken. A study on the combined synergistic efficacy between Kava kava and Passiflora was undertaken where there was a significant sedative prolongation with combined use (Capasso and Sorrentino, 2005). *Panax ginseng* with *Ginkgo biloba* was shown to have improved cognitive function when combined (Scholey and Kennedy, 2002). More specifically, in spite of the many anecdotal accounts of plants used in combination for the treatment of microbe related infections, very few *in vitro* antimicrobial combination studies have been undertaken to validate the role of antimicrobial synergism in phytotherapy. Some documented accounts include *Portulaca quadrifida* with *Monadenium lugardiae* to treat stomach complaints; *Trichilia emetica* with *Cyathula natalensis* for leprosy and *Momoridica foetida* with *Pittosporum viridiflorum* for boils (Hutchings *et al*., 1996). A follow-up literature search yielded only three studies on plants used

in combination for antimicrobial therapy. A disc diffusion study was undertaken by Hsieh *et al*., (2001) where *Cinnamomum cassia* was combined with *Allium tuberosum* and the fruit of *Cornus officinalis* at different ratios. Results indicated combined efficacy at various ratio combinations. A more recent study on the combined effect of *Origanum vulgare* and *Vaccinium macrocarpon* was undertaken using the disc diffusion assay, optical density evaluation and colony counts. After treatment over a number of days, efficacy was enhanced in combination (Lin *et al.*, 2005). Another combination study using isolobolograms to plot the combined MIC in relation to the MIC as determined independently was undertaken with *Salvia chamelaeagnea* and *Leonotis leonurus.* The ethnobotanical use of *S. chamelaeagnea* and *L. leonurus* in combination to treat respiratory infections was validated (Kamatou *et al.*, 2006). While these studies cannot be directly compared to the time-kill efficacy examined here, they do support the hypothesis that synergy between plants of different species exist.

5.7 General conclusions

- The major constituents of the essential oil of *Lippia javanica* are linalool (65.2%), and (*Z*)-β-ocimene (13.0%), representing 78.2% accumulatively.

- The highest antimicrobial activities obtained by means of the disc diffusion assay were noted for *Klebsiella pneumoniae* (5 mm).

- The highest antimicrobial activity noted in the MIC methodology was against *Escherichia coli* and *Cryptococcus neoformans* (4 mg/mL).

- Death kinetic assays for the three organisms studied showed that the killing rate was the greatest for *Klebsiella pneumoniae*, thereafter *Cryptococcus neoformans* and very little reduction of microbial populations for *Bacillus cereus.*

- The ethnobotanical use of *Lippia javanica* to treat dysentery, diarrhoea and suspected food poisoning is validated by the highest antimicrobial activity noted in the MIC methodology against *Escherichia coli.*

- The ethnobotanical use of *Lippia javanica* to treat coughs, colds, bronchial and respiratory ailments is validated by the highest efficacies obtained in the MIC methodology, time-kill assays as well as the use in combination with *Artemisia afra*.

- The time-kill study for *Lippia javanica* with *Artemisia afra* indicates that the oils in combination act synergistically against the test organism *Klebsiella pneumoniae*.

Chapter 6

Helichrysum cymosum (L.) D. Don subsp. *cymosum*, antimicrobial activity of the essential oil, extract and isolated bioactive compound helihumulone.

6.1 Introduction

The genus *Helichrysum*, belonging to the Asteraceae family consists of approximately 500 species (Bougatso *et al.*, 2004). South Africa harbours in the region of 245 species of *Helichrysum* and biological studies have been performed on a number of these species. Of the many species that have been investigated (Hutchings and van Staden, 1994; Meyer and Dilika, 1996; Afolayan and Meyer, 1997; Dilika *et al.*, 1997; Mathekga and Meyer, 1998; Lourens *et al.*, 2004; Scott *et al*., 2004; Yani *et al*., 2005) *Helichrysum cymosum* D. Don subsp. *cymosum* has received very little attention. A non-quantitative antimicrobial screening has been reported for the ethanol extracts of *H. cymosum* where activities were selectively observed for Gram-positive organisms only (Sindambiwe *et al*., 1999). More recently (Bougatso *et al*., 2004; van Vuuren *et al.*, 2006) reported the composition of the essential oil and antimicrobial activity. Up until now, the research focus of this thesis has been on the volatile components of aromatic plants used in traditional healing rites. However, one must take cognizance that non-volatile compounds may be responsible for antimicrobial efficacy and one should not focus on the volatile fraction only when studying aromatic plants. With this in mind, the study of *H. cymosum* subsp. *cymosum* was undertaken to additionally investigate the activity of the non-volatile compounds not present in the essential oils.

6.2 Botanical description

Members of the genus *Helichrysum* are usually aromatic, perennial shrubs, having dense leaves with hardy yellow flower heads (Figure 6.1). The genus is characterized by hairy leaves and persistent flower heads, which when dried keep their shape for years (van Wyk *et al*., 1997). The word *Helichrysum* is derived from the Greek word “helios” meaning sun and “chrysos” meaning gold, referring to the distinctive flowers of the genus.



Figure 6.1 *Helichrysum cymosum* subsp. *cymosum* in habitat.

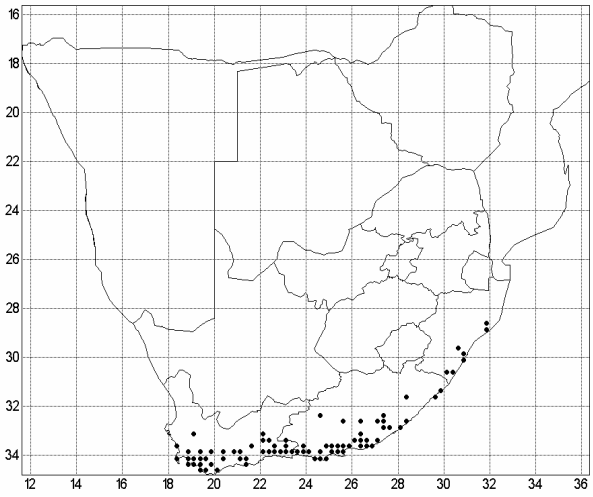


Figure 6.2 Geographical distribution of *H. cymosum* subsp. *cymosum* in South Africa (SANBI).

6.3 Distribution

Figure 6.2 illustrates the geographical distribution of *H. cymosum* subsp. *cymosum* in South Africa. The species is distributed mainly along the south and easterly coastal regions from the Western Cape through to KwaZulu-Natal.

6.4 Medicinal uses

The medicinal uses of *Helichrysum* spp. have been widely reported. Extracts of various species have been used to treat topical infections, respiratory ailments and have been incorporated in dressings used after circumcision rites (Meyer and Dilika, 1996; Mathekga and Meyer, 1998). Administration by inhalation suggests that volatile compounds may play a role in antimicrobial therapy and several studies indicate significant antimicrobial properties for *Helichrysum* oils (Hutchings and van Staden, 1994; Roussis *et al*., 2000). The traditional use as an analgesic and anti-inflammatory suggests that it may be used for the treatment of other tropical diseases such as malaria (Hutchings and van Staden, 1994; van Vuuren *et al*., 2006). Of all the *Helichrysum* species found in South Africa, *H. cymosum* subsp. *cymosum* is among the best known and most commonly used in traditional healing practices (Bougatso *et al*., 2004). The route of administration varies from inhalation of burned leaves (possibly suggesting active volatile

constituents) to oral ingestion of a tea preparation (possibly extracting more polar non-volatile constituents), with extracts applied to topical infections.

6.5 Methods

6.5.1 *Chemical aspects*

Plant collection and distillation of essential oils: Plant material was harvested from the wild, during April near Oudtshoorn, one kilometre east of Robinsons Pass. A voucher specimen (Table 6.1) is deposited in the Department of Pharmacy and Pharmacology, University of Witwatersrand. The plants were hydrodistilled as described in Chapter 2.5.1.

Table 6.1 Plant collection data for *H. cymosum* subsp. *cymosum.*

Plant	Voucher	Material distilled (g)	Essential oil yield (% w/w)
H. cymosum subsp. *cymosum*	JV790	1440	0.2

Gas chromatography combined with mass spectrometry (GC-MS) data: Oil samples were analyzed with GC-MS using the Hewlett-Packard 1800A GCD system operating under the same conditions as described in Chapter 2.5.1.

6.5.1.1 Isolation of antimicrobial compound

Extraction: The extracts were prepared by submerging the dried macerated plant material (297 g) in acetone overnight. Plant material was retained at room temperature and thereafter filtered. The extraction solvent was evaporated at 60°C using a rotary evaporator (Büchi) for approximately three hours.

Bio-autographic assays: Thin layer bio-autographic assays (Marston and Hostettmann, 1999) were carried out by placing 5 µl of the acetone extract (50 mg/mL) on silica gel thin layer chromatography (TLC) plates (Alugram ® Sil G/UV_{254}, 0.2 mm). The plates were eluted with toluene 72%, dioxane 20% and acetic acid 8% (Merck). Two TLC plates were prepared under identical conditions, one to be incubated with the test organism *S. aureus* and Tryptone Soya agar, and a reference plate without test organism and agar, kept at ambient temperature. A base

layer (15 mL) of Tryptone Soya agar was poured into a sterile petri dish. The developed TLC plate was dried and sterilized under ultra violet (UV) light at 254 nm for one hour. Thereafter it was placed onto the agar surface with the silica side facing upwards. An overlay of Tryptone Soya agar (15 mL) containing a bacterial culture *S. aureus,* ATCC 12600 (approximate inoculum size, 1 x 10^6 CFU/mL) was placed on top of the TLC plate. The plates were allowed to prediffuse at 4 °C for one hour prior to incubating at 37 °C for 24 hr. The resulting inhibition zones were visualized around active compounds by spraying with 0.04% (w/v) INT and compared to the reference TLC plate (Figure 6.3). The acetone extract of *H. cymosum* subsp. *cymosum* was subsequently subjected to bioassay-guided fractionation.

Bioactivity-guided fractionation and isolation using column chromatography: Column chromatography was used as a purification technique to isolate the antimicrobial compound. Several solvent systems were prepared and run with the crude acetone extract to determine the ideal mobile phase, of which 100% chloroform (Merck) was selected. The extract (4.8 g) was dissolved in chloroform to produce a slurry. The column was prepared by plugging the tap with cotton wool and filling the column with silica gel 60 (0.063-0.2 mm / 70-230 mesh ASTM, Macherey-Nagel). The slurry (extract together with chloroform) was applied to the top of the column. Four consecutive columns, packed with silica gel 60 and eluted with 100% chloroform were prepared. With each column, fractions were collected, and samples run on TLC which was subsequently analyzed by viewing the chromatograms under UV light at 254 nm and 366 nm. Similar fractions were combined and concentrated to dryness with the aid of a rotary evaporator. Combined fractions were subjected to bio-autographic assays against *S. aureus* (Figure 6.4). The bioactive fractions were further purified on Sephadex LH-20 (Sigma-Aldrich) eluted with methanol to yield 277 mg of an isolated biologically active compound. Figure 6.5 summarizes the bioactivity-guided fractionation procedure.

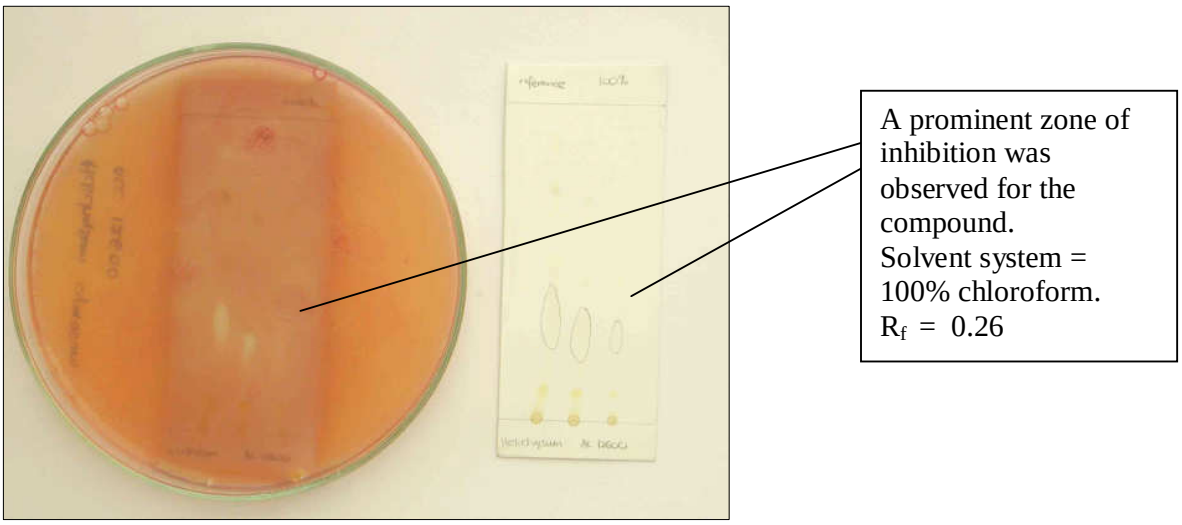


Figure 6.3 Bio-autographic assay of the crude acetone extract of *H. cymosum* subsp. *cymosum* against *S. aureus*.

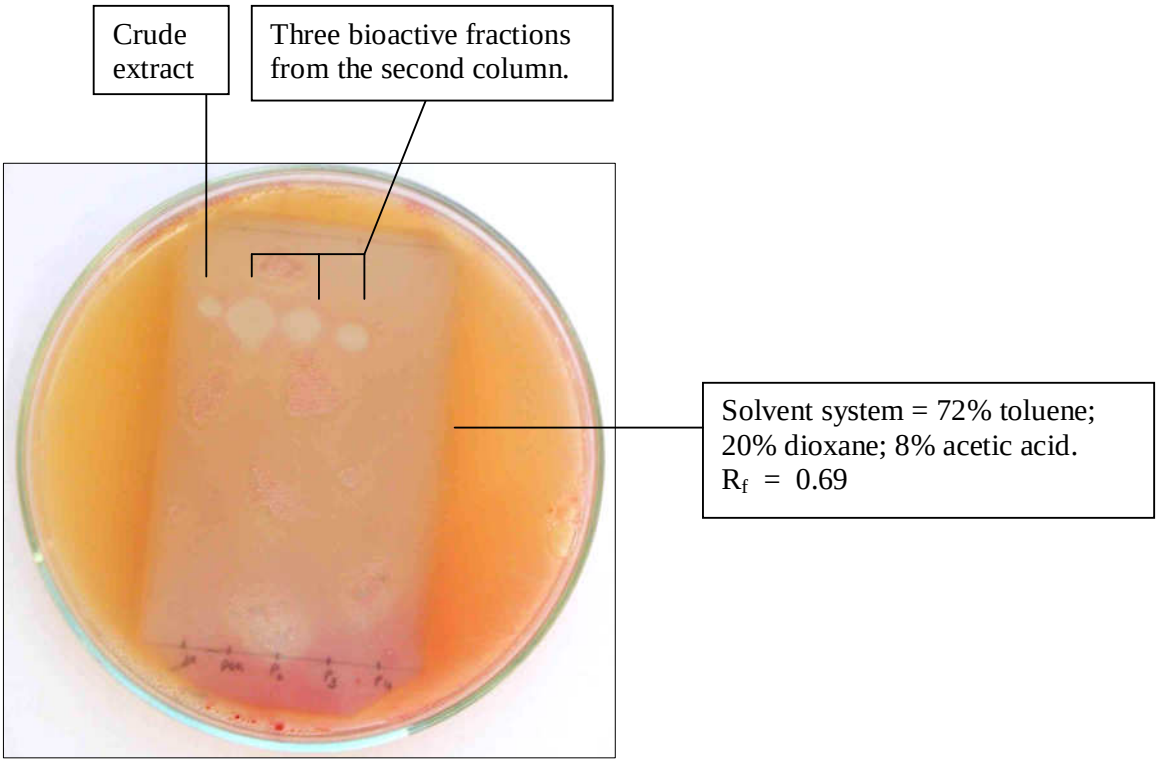
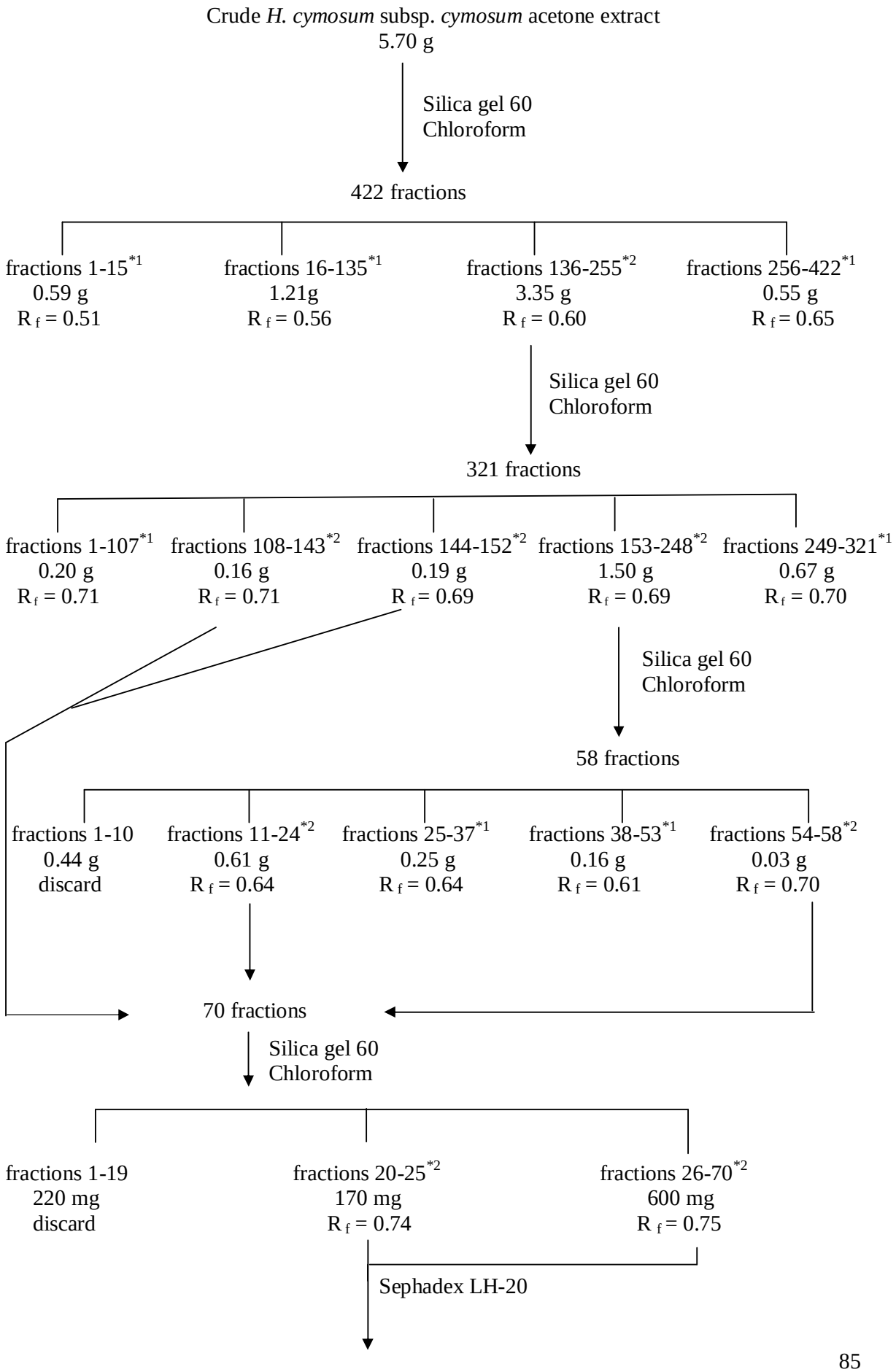


Figure 6.4 Follow-up isolation of the biologically active compound by assay-guided fractionation using *S. aureus* as a test pathogen.


Crude *H. cymosum* subsp. *cymosum* acetone extract
5.70 g
Silica gel 60
Chloroform
422 fractions
fractions 1-15*1
0.59 g
R f = 0.51
fractions 16-135*1
1.21g
R f = 0.56
fractions 136-255*2
3.35 g
R f = 0.60
fractions 256-422*1
0.55 g
R f = 0.65
Silica gel 60
Chloroform
321 fractions
fractions 1-107*1
0.20 g
R f = 0.71
fractions 108-143*2
0.16 g
R f = 0.71
fractions 144-152*2
0.19 g
R f = 0.69
fractions 153-248*2
1.50 g
R f = 0.69
fractions 249-321*1
0.67 g
R f = 0.70
Silica gel 60
Chloroform
58 fractions
fractions 1-10
0.44 g
discard
fractions 11-24*2
0.61 g
R f = 0.64
fractions 25-37*1
0.25 g
R f = 0.64
fractions 38-53*1
0.16 g
R f = 0.61
fractions 54-58*2
0.03 g
R f = 0.70
70 fractions
Silica gel 60
Chloroform
fractions 1-19
220 mg
discard
fractions 20-25*2
170 mg
R f = 0.74
fractions 26-70*2
600 mg
R f = 0.75
Sephadex LH-20

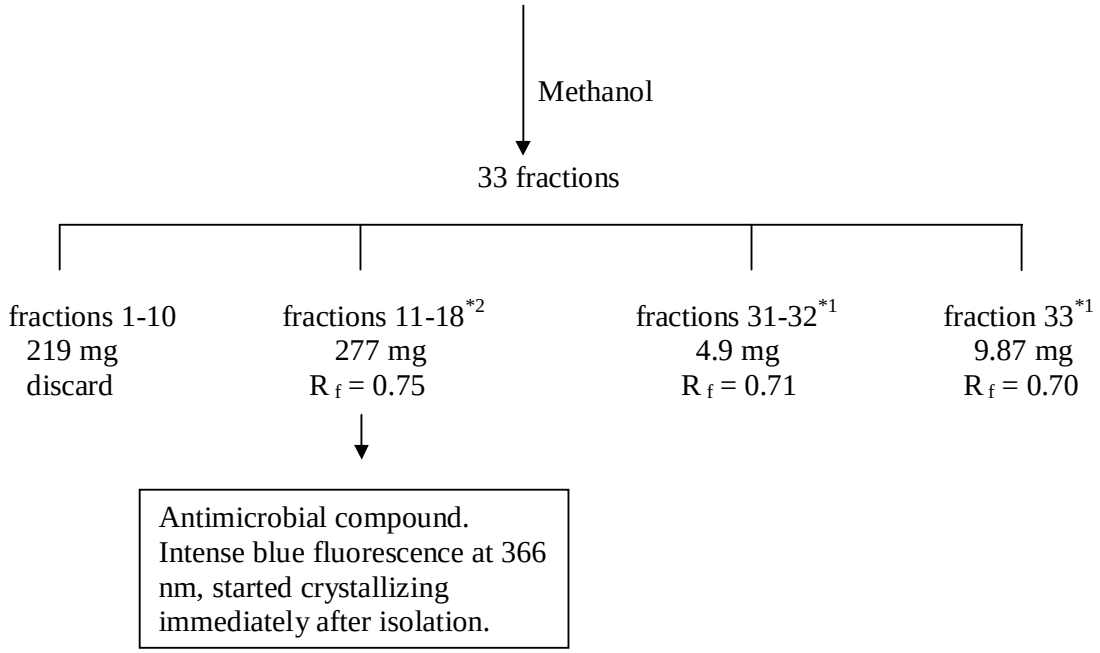


Figure 6.5 Schematic representation of the bioactivity guided fractionation procedure.

*1 No antimicrobial activity was noted on further bio-autography investigations
*2 Antimicrobial activity was noted for *S. aureus* on further bio-autography investigation.

Identification and elucidation of isolated compounds: The isolated compound was subjected to nuclear magnetic resonance spectrometry (NMR) and MS for identification in collaboration with the Department of Chemistry and Biochemistry at the University of Johannesburg. Spectra were recorded on a Varian Inova 2000 300 MHz spectrometer. All spectra were recorded at 25 °C in deuterated chloroform and the chemical shifts were recorded in ppm referenced to tetramethylsilane or the solvent shift. Electron ionization MS of the compound was performed by direct inlet at 70 eV on the GCMS-QP2010 gas chromatography-mass spectrometer.

The crude extract constituents were analysed by high performance liquid chromatography (HPLC) coupled to MS and using a Waters 2690 HPLC system (Phenomenex Aqua C18 column, 250 mm x 2.1 mm at 40 °C) and equipped with a 996 photodiode array detector (PDA) and Thermabeam mass selective detector (TMD). The samples were dissolved in methanol at a concentration of 50 mg/mL and 10 µl injected under the following conditions: mobile phase flow rate; 0.2 mL/min, nebulizer gas flow; 30 L/h, nebulizer temperature; 80 ˚C, expansion region; 90 ˚C and source temperature; 225 ˚C. The initial mobile phase was acetonitrile, 90% water containing 100 mM formic acid and the solvent ratio was changed through a linear gradient to 90% acetonitrile, 10% water with 100 mM formic acid at 40 min. This ratio was maintained for

10 min after which the solvent ratio was converted to that of the initial starting conditions. Data analysis was performed using the Empower® software program.

6.5.2 Antimicrobial aspects

Culture, media preparation and disc diffusion assays were undertaken according to the NCCLS (2003) guidelines and additional references as described in Chapter 2.5.2. Preliminary antimicrobial screening by disc diffusion assays was performed with the essential oil and the acetone extract on ten reference strains: *Yersinia enterocolitica, E. faecalis, B. cereus, B. subtilis, S. aureus, P. aeruginosa, E. coli, K. pneumoniae, C. albicans* and *C. neoformans.*

The MIC was determined using the micro-dilution bioassay method as in Chapter 2.5.2, where the essential oil, crude acetone extract and the isolated bioactive fraction were examined against the same ten pathogens as investigated in the disc diffusion assay. Two strains were introduced which were not studied in Chapters 2-5. The acceptable control ranges for ciprofloxacin against these additional pathogens used in the MIC assays are given in Table 6.2. The starting concentration for the essential oil was 128 mg/mL. The starting concentration for the extract and bioactive fraction for MIC determination was 64 mg/mL and 5 mg/mL respectively.

Table 6.2 Acceptable MIC ranges for ciprofloxacin control.

Test organism	MIC controls (μg/mL)*
Yersinia enterocolitica (ATCC 23715)	0.39-1.00
Klebsiella pneumoniae (ATCC 13883)	0.12-0.80

* Acceptable ranges as determined within the Department of Pharmacy and Pharmacology, University of the Witwatersrand (controls in this study were run for all test organisms).

6.6 Results and discussion

6.6.1 Essential oil chemistry

Sixty-five compounds were identified in the *H. cymosum* subsp. *cymosum* essential oil representing 85.2% of the total composition (Table 6.3).

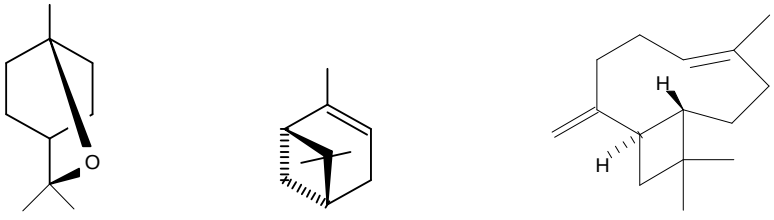
Table 6.3 Essential oil composition of *H. cymosum* subsp. *cymosum*.

RRI*	Compound name	Area percentage
1032	**α-pinene**	**12.4**
1072	α-fenchene	0.1
1076	Camphene	0.4
1118	β-pinene	3.7
1132	Sabinene	0.2
1174	Myrcene	1.2
1188	α-terpinene	1.2
1203	Limonene	7.2
1213	**1,8-cineole**	**20.4**
1246	(*Z*)-β-ocimene	9.5
1255	γ-terpinene	1.4
1266	(*E*)-β-ocimene	3.6
1280	p-cymene	2.4
1290	Terpinolene	0.4
1320	2-heptanol	0.1
1386	1-hexanol	tr
1382	*cis*-allo ocimene	0.4
1391	(*Z*)-3-hexen-1-ol	0.1
1398	2-nonanone	0.4
1426	2-octanol	0.5
1452	1-octen-3-ol	0.3
1493	α-ylangene	0.1
1497	α-copaene	1.20
1521	2-nonanol	0.1
1535	β-bourbonene	0.6
1541	Benzaldehyde	0.1
1544	α-gurjunene	0.1
1553	Linalool	0.1
1586	pinocarvone	0.1
1591	fenchylalcohol	0.7
1612	**β-caryophyllene**	**10.8**
1628	aromadendrene	1.5
1631	selina-5,11-diene	0.3
1664	*trans*-pinocarveol	0.6
1682	δ-terpineol	0.9
1687	α-humulene	1.3
1700	Limonene-4-ol	0.2
1706	α-terpineol	2.6
1719	Borneol	1.8
1730	δ-guaiene	1.1
1740	α-muurolene	0.3

RRI*	Compound name	Area percentage
1773	δ-cadinene	0.6
1804	myrtenol	0.6
1838	2-phenyl ethyl acetate	tr
1845	*trans*-carveol	0.2
1853	*cis*-calamenene	0.6
1864	p-cymen-8-ol	0.1
1889	benzylacetone	0.3
1896	*cis*-p-mentha-1(7), 8-dien-2-ol	tr
1941	α-calacorene	0.1
2001	isocaryophyllene oxide	0.1
2008	caryophyllene oxide	1.6
2033	epiglobulol	0.3
2071	humulene epoxide II	0.2
2073	β-caryophyllene alcohol	0.4
2098	globulol	0.6
2104	viridiflorol	0.8
2144	rosifoliol	0.2
2184	*cis*-p-menth-3-en-1,2-diol	0.1
2204	clovenol	0.1
2255	α-cadinol	0.1
2316	caryophylladienol I	tr
2324	caryophylladienol II	0.4
2389	caryophyllenol I	0.2
2392	caryophyllenol II	0.1
	TOTAL	**85.2**

*RRI: Relative retention indices as eluted from a polar column.
% calculated from TIC data
tr = Trace <0.1%

Major compounds: The chemical constituents of the essential oil (Table 6.3) analyzed by GC-MS indicate the major compounds of *H. cymosum* subsp. *cymosum* (Figure 6.6) as α-pinene (12.4%), 1,8-cineole (20.4%) and β-caryophyllene (10.8%) comprising 43.6% of the total oil composition.



1,8-cineole α-pinene β-caryophyllene

Figure 6.6 Chemical structures for major compounds identified in the essential oil of *H. cymosum* subsp. *cymosum*.

6.6.2 Elucidation and identification of the isolated compound

Based on ^{1}H, ^{13}C, COSY, HMQC and HMBC experiments, the compound was identified as the known helihumulone, a phloroglucinol-derived compound previously isolated from *H. cymosum* (Bohlmann *et al*., 1979). The chemical shift data (Table 6.6) as well as the carbon (^{13}C, Figures 6.8) and proton (^{1}H, Figure 6.9) NMR spectra for helihumulone can be found as an appendix to this Chapter.

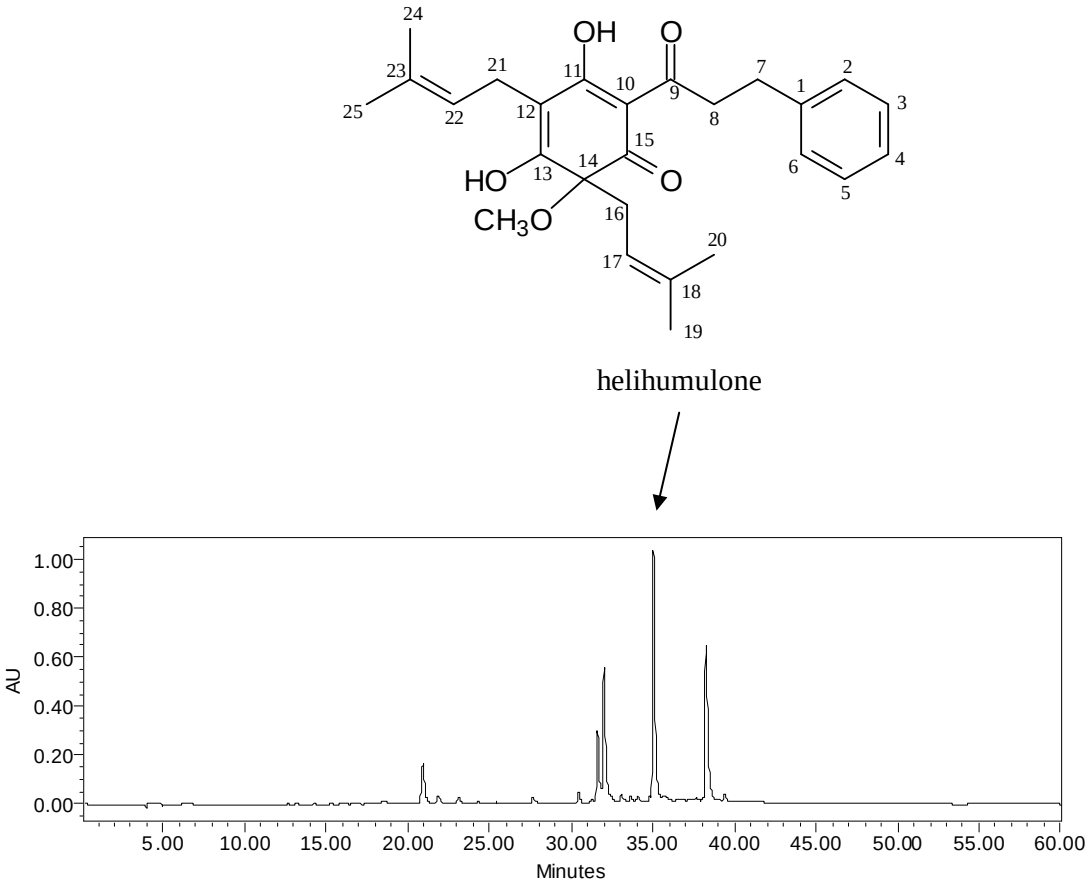


Figure 6.7 The HPLC profile of *H. cymosum* subsp. *cymosum* with the major peak identified as helihumulone.

The HPLC profile (Figure 6.7) indicates a single major compound. Based on ^{1}H, ^{13}C, COSY, HMQC and HMBC experiments, this compound was identified as the known helihumulone, a phloroglucinol-derived compound previously isolated from *H. cymosum* subsp. *cymosum* (Bohlmann *et al*., 1979).

6.6.3 Antimicrobial activity

The preliminary disc diffusion results (Table 6.4), indicated good activities only for the Gram-positive organisms when exposed to the acetone extracts.

Table 6.4 Zones of inhibition (measured in mm from edge of disc) of the acetone extract and essential oil of *H. cymosum* subsp. *cymosum*.

Pathogen	Extract	Oil	Control*
Enterococcus faecalis (ATCC 292192)	3.7	0	4.0
Bacillus cereus (ATCC 11778)	7.0	0	6.0
Bacillus subtilis (ATCC 6051)	5.7	0	11.0
Staphylococcus aureus (ATCC 25923)	8.0	0	3.0
Pseudomonas aeruginosa (ATCC 9027)	0	0	3.0
Escherichia coli (ATCC 11775)	0	0	5.0
Yersinia enterocolitica (ATCC 23715)	0	0	5.0
Klebsiella pneumoniae (ATCC 13883)	0	0	6.0
Cryptococcus neoformans (ATCC 90112)	0	0	5.0
Candida albicans (ATCC 10231)	0	0	5.0

* Neomycin and nystatin served as controls for bacteria and yeasts respectively.

On further bio-autographic investigation of the crude acetone extract, a single prominent zone of inhibition was observed for the compound with a R_f value of 0.26 when tested against *S. aureus* (Figure 6.3) thus suggesting that a single compound could possibly be responsible for antimicrobial activity in this species. Helihumulone has previously been isolated from *H. cymosum* (Bohlmann *et al*., 1979), however, no report was made on any of the biological activities of the compound. Helihumulone was further quantitatively examined for antimicrobial activity by the MIC assay together with the essential oil and acetone extract (Table 6.5). Even though antimicrobial activity was only selectively found in the disc diffusion assay, MIC evaluations were performed for all test pathogens, as false negatives, often associated with disc diffusion assays may have occurred. This method variation is further discussed in Chapter 11.

Table 6.5 MIC (mg/mL) for *H. cymosum* subsp. *cymosum* essential oil, acetone extract and helihumulone against ten pathogens.

Pathogen	Essential oil	Extract	Helihumulone	Control*
Enterococcus faecalis (ATCC 292192)	1.000	0.156	0.031	2.0×10^{-3}
Bacillus cereus (ATCC 11778)	8.000	0.078	0.063	1.0×10^{-3}
Bacillus subtilis (ATCC 6051)	8.000	0.313	0.063	3.0×10^{-3}
Staphylococcus aureus (ATCC 25923)	8.000	0.313	0.125	0.5×10^{-3}
Pseudomonas aeruginosa (ATCC 9027)	4.000	0.313	0.016	1.2×10^{-3}
Escherichia coli (ATCC 11775)	8.000	0.313	0.063	0.1×10^{-3}
Yersinia enterocolitica (ATCC 23715)	8.000	0.313	0.063	1.0×10^{-3}
Klebsiella pneumoniae (ATCC 13883)	8.000	0.313	0.063	0.2×10^{-3}
Cryptococcus neoformans (ATCC 90112)	4.000	0.078	0.031	1.0×10^{-3}
Candida albicans (ATCC 10231)	4.000	0.313	0.063	2.5×10^{-3}

* Ciprofloxacin and amphotericin B served as controls for bacteria and yeasts respectively.

The MIC values (mg/mL) ranges from 1 - 8 (essential oil), 0.078 - 0.313 (crude extract) and 0.016 - 0.125 (helihumulone) indicating that the crude extract is at least six times more antimicrobially active than the essential oil, and similarly the isolated compound is more active than the crude extract. The highest sensitivity for the essential oils was against *E. faecalis* (1 mg/mL). This pathogen is associated with respiratory disorders and could possibly be correlated with the traditional inhalation use of *H. cymosum.* Previous studies on the antimicrobial activity of α-pinene, a major compound (12.4%) found in the essential oil of *H. cymosum* have shown moderate (Knobloch *et al*., 1989; Dorman and Deans, 2000; Tzakou *et al*., 2001) to poor activity (Hinou *et al*., 1989; Moleyar and Narasimham, 1992; Griffin *et al*., 1999; van Zyl *et al*., 2006). Thus, it is probable that this compound does not play a major role in the antimicrobial efficacy of the plant. The highest sensitivities for the extracts were against *B. cereus* and *C. neoformans* (78 µg/mL). The highest sensitivity for the bioactive compound helihumulone may be attributed to *P. aeruginosa* (31 µg/mL), a pathogen often associated with wound infections. The numerous accounts (Watt and Breyer-Brandwijk, 1962; Hutchings *et al*., 1996; Meyer and Dilika, 1996; Mathekga and Meyer, 1998) of the *Helichrysum* genus as a dressing for wounds is validated by the high efficacies noted for *P. aeruginosa.* With the exception of the activity for *S. aureus* (125 µg/mL), helihumulone indicated good activity with values less than 64 µg/mL. A review publication on anti-staphylococcal compounds (Gibbons, 2004) has suggested that only compounds with a MIC value of less than 64 µg/mL have clinical antimicrobial significance.

Considering this statement one can therefore deduce that the antimicrobial activity of helihumulone was significant for nine of the ten pathogens studied.

No antimicrobial activity was found in the essential oil study of the Tanzanian *H. cymosum* species (Bougatso *et al*., 2004) when investigated against nine test micro-organisms. No starting concentrations were given in the methodology and therefore comparative examination cannot be made, as starting concentrations may have been lower than the MIC's obtained in this study. Major compounds in the Tanzanian *H. cymosum* essential oil were *trans*-caryophyllene (27.02%) and *p*-cymene (7.55%). α-Pinene, a major compound found in the essential oil of this study (12.4%) was detected only as a minor compound in the Tanzanian *H. cymosum* essential oil (0.75%). Also 1,8-cineole (20.4%), a major compound in this study is absent in the Tanzanian *H. cymosum* essential oil. From these essential oil comparisons it is not surprising that different biological activities were noted. As noted in previous studies (Griffin *et al*., 2000; Dorman and Deans, 2000), the antimicrobial activity is merely an expression of the chemical composition and activity is dependent on the structural configuration.

These results highlight the importance of examining both essential oils and extracts when the bioactivities of aromatic plants are investigated. As was noted, the highest antimicrobial activity can be attributed to helihumulone isolated from the crude extract. Helihumulone is not found in the essential oil, which could possibly indicate why the essential oil showed lower antimicrobial activities than the crude extract.

6.7 General conclusions

- The major constituents of the essential oil of *Helichrysum cymosum* subsp. *cymosum* are α-pinene (12.4%), 1,8-cineole (20.4%) and β-caryophyllene (10.8%).

- Using bioassay-guided fractionation, the most active compound was isolated with column chromatography and identified as the known phloroglucinol derivative, helihumulone.

- The MIC ranges for the essential oil were 1– 8 mg/mL with the highest activity noted for the respiratory pathogen *Enterococcus faecalis*, thus validating the traditional use of the plant to treat respiratory ailments.

- The MIC ranges for the crude extract were 0.078 – 0.313 mg/mL with highest activity noted for *Bacillus cereus* and *Cryptococcus neoformans*.

- The MIC ranges for the isolated compound helihumulone were 0.016 – 0.125 mg/mL with the highest activity noted for *Pseudomonas aeruginosa*, thus validating the traditional use of the plant for wound dressings.

Appendix

Helichrysum cymosum subsp. *cymosum*

Table 6.6 Correlation of NMR chemical shift data ^{13}C and ^{1}H for helihumulone with literature values obtained by Bohlmann *et al*. (1979).

Position	^{13}C NMR data δ_C	^{13}C NMR literature	^{1}H NMR data δ_H	^{1}H NMR data literature
1	141.0*s*[*1]	141.0*s*		
2,6	128.2	128.5*d*	7.25*m*[*5]	7.27*m*
3,5	128.2	128.4*d*	7.25*m*	7.35*m*
4	126.1*d*[*2]	126.1*d*	7.25*m*	
7	39.5*t*[*3]	39.7*t*	2.95*m*	2.95*t*
8	41.2*t*	42.1*t*	3.30*m*	
9	201.2*s*	201.3*s*		
10	112.9*s*	113.3*s*		
11	190.2*s*	190.4*s*		
12	108.0*s*	108.3*s*		
13	166.9*s*	166.4 *s*		
14	84.0*s*	84.7*s*		
15	192.3*s*	192.5*s*		
16	31.1*t*	31.3*t*	2.54*m*	2.54*dd*
17	115.0*d*	115.4*d*	4.88*m*	4.81*t*(*br)*
18	137.8*s*	138.0*s*		
19	17.6*q*[*4]	17.8*q*	1.49	1.59*s*(*br*)
20	25.7*q*	25.8*q*	1.60	1.55*s*(*br*)
21	21.1*t*	21.3*t*	3.10*m*	3.19*d*(*br*)
22	121.1*d*	121.3*d*	5.12*m*	5.15*t*(*br*)
23	132.5*s*	132.4*s*		
24	17.6*q*	17.8*q*	1.69	
25	25.6*q*	25.7*q*	1.74	1.79*s*(*br*)
OMe	53.9*q*	54.0*q*	3.15	

[*1] singlet; [*2] doublet; [*3] triplet; [*4] quartet; [*5] multiplet

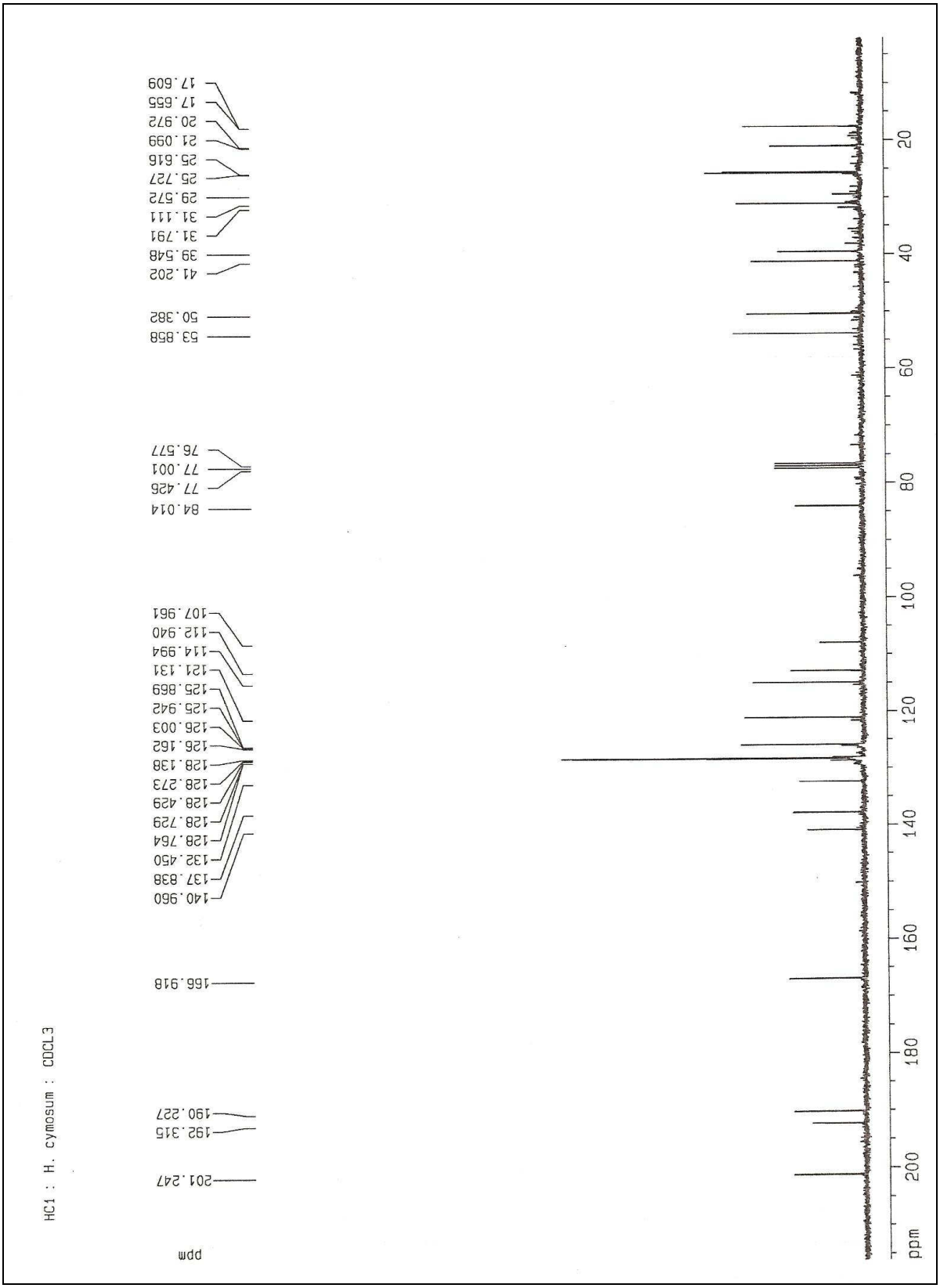


Figure 6.8 The NMR carbon spectra (^{13}C) for helihumulone.

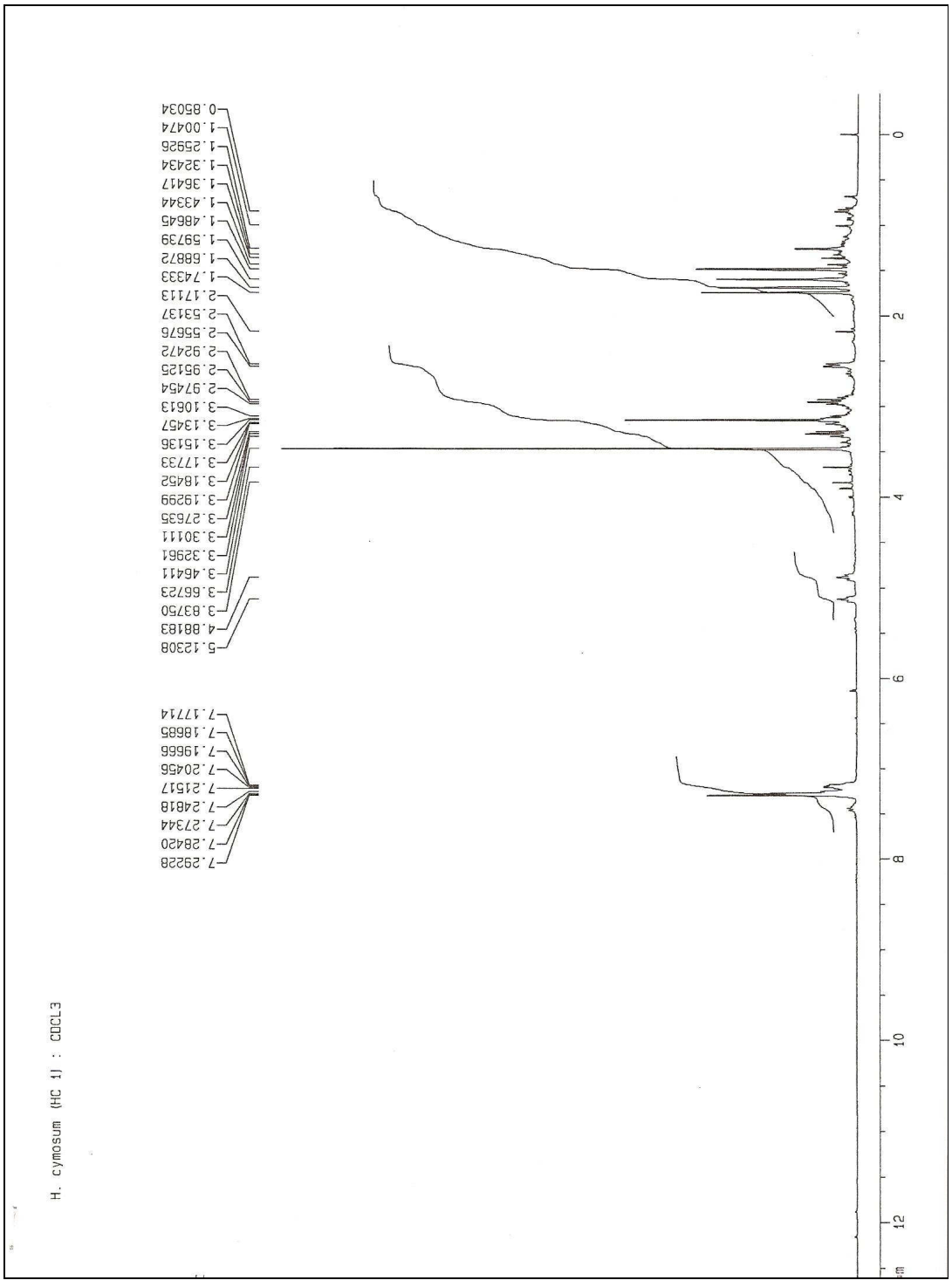


Figure 6.9 The NMR proton spectra (^{1}H) for helihumulone.

Chapter 7

Croton gratissimus Burch. var. *subgratissimus*, the antimicrobial activity of the essential oil and extracts, with evidence of synergy for plant part combinations.

7.1 Introduction

Despite the traditional use of the genus *Croton* for its medicinal properties (Watt and Breyer-Brandwijk, 1962; van Wyk *et al.*, 1997; van Wyk and Gericke, 2000), scientific studies validating the therapeutic properties of the indigenous species *Croton gratissimus* Burch. var. *subgratissimus* (from the family Euphorbiaceae) are lacking. The leaves of this tree are aromatic and often used in herbal preparations and may also be used with the bark and roots (Hutchings *et al.*, 1996; van Wyk *et al.*, 1997; von Koenen, 2001). It has been suggested when investigating aromatic plants that one should not restrict research only to the volatile essential oils (van Vuuren *et al.*, 2006). This was noted in the study on *H. cymosum* subsp. *cymosum* (Chapter 6) where the antimicrobial spectrum of activity for extracts was much higher than that for the essential oils. There are numerous ethnobotanical reports (Watt and Breyer-Brandwijk, 1962; Hutchings *et al.*, 1996; van Wyk *et al.*, 1997; von Koenen, 2001; Ngwenya *et al*., 2003) of plants used in combination therapy i.e. where plant parts such as roots and leaves are combined and used in therapeutic regimens. *Croton* is one such plant where the bark, roots and leaves are used independently as well as in combination. This study focused on the antimicrobial potential of *C. gratissimus* var. *subgratissimus* and the interactive role of the different plant parts when used in combination for antimicrobial therapy in order to validate the traditional use.

7.2 Botanical description

This tree can grow up to ten meters in height. Leaves are distinctive (Figure 7.1) as they are dark green on the upper side and have a shiny gray to silver coloration on the lower surface (van Wyk *et al*., 1997). The under side of the leaf is scattered with dark brown glands. The bark is rough and gray in colour. Cream-coloured flowers are produced in autumn, which yield small fruit capsules (van Wyk *et al*., 1997).



Figure 7.1 The leaves and fruit of *Croton gratissimus* var. *subgratissimus*.

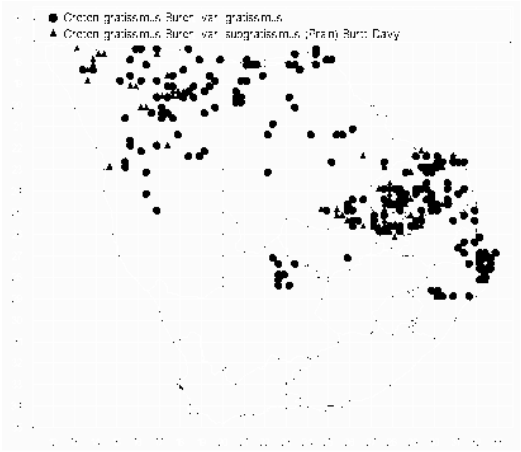


Figure 7.2 The geographical distribution of *Croton gratissimus* var. *subgratissimus* in southern Africa. (SANBI).

7.3 Distribution

Figure 7.2 illustrates the geographical distribution of *C. gratissimus* var. *subgratissimus* in southern Africa. The tree occurs naturally over a large area (Northern Cape, North-West Province, Gauteng, northern KwaZulu-Natal, Mpumalanga and Northern Province) and in the northern parts of South Africa (van Wyk *et al*., 1997). A number of populations have been recorded in central to northern Botswana and Namibia.

7.4 Medicinal uses

The Afrikaans name for the plant “koorsbessie” (“koors” = fever) suggests that these trees are used as a pyrogenic. All parts of the plant have reputed medicinal value. Leaf infusions are used for coughs and placed in steam baths to reduce fever (van Wyk *et al.*, 1997; Hutchings *et al.*, 1996). Eye lotions are made for livestock from cold infusions of the leaf (Watt and Breyer-Brandwijk, 1962). A deconcoction of the roots has been used for chest complaints, coughs, fever and sexually transmitted diseases such as syphilis (von Koenen, 2001). The bark has the highest reported use including the treatment of bleeding gums, abdominal disorders, skin inflammation, earache and chest complaints, suggesting microbial infections may be responsible (Hutchings *et al.*, 1996; von Koenen, 2001). Various combinations of *C. gratissimus* have been used. The

combination of roots and bark to treat respiratory disorders has been reported (von Koenen, 2001). Accounts of the administration with other species (Hutchings *et al.*, 1996) have been noted i.e. for the treatment of swellings where the bark of *C. gratissimus* is combined with the root of Amaryllidaceae species and rubbed into incisions. Also noted, was the use of the bark of *C. gratissimus* together with the bark of *Ocotea bullata* where powdered forms are blown into the womb to treat uterine disorders. Even though the plant is considered toxic, the very nature of the irritancy action may serve as a purgative or eruptive emetic thus ridding the body of toxins and microbial infection.

7.5 Methods

7.5.1 *Chemical aspects*

Plant collection and distillation of essential oils: The leaf, bark and root samples (Figure 7.3) used in this study was collected from a single tree in the wild on the western slopes below Diep-in-die Berg, in the Tshwane area during May. A voucher specimen (Table 7.1) is deposited in the Department of Pharmacy and Pharmacology, University of Witwatersrand. Leaf material was hydrodistilled, as described in Chapter 2.5.1.



Figure 7.3 The leaves, bark and root of *C. gratissimus* var. *subgratissimus*.

Table 7.1 Plant collection data for *C. gratissimus* var. *subgratissimus*.

Plant	Voucher	Material distilled (g)	Essential oil yield (% w/w)
C. gratissimus var. *subgratissimus*	PB975	470.8	0.3

Gas chromatography combined with mass spectrometry (GC-MS) data: Oil samples were analyzed with GC-MS using the Hewlett-Packard 1800A GCD system operating under the same conditions as described in Chapter 2.5.1.

Preparation and analysis of the crude extracts: Extracts of the leaves, bark and roots were prepared by submerging the dried macerated plant material in a 1:1 mixture of methanol (Merck) and chloroform (Merck) overnight. Plant material was retained at ambient temperature and thereafter filtered. The solvent was evaporated at 60 °C using a rotary evaporator for approximately three hours.

High performance liquid chromatography (HPLC) analysis: In order to determine if any major chemical variation between subterranean and above ground foliage exists, HPLC analysis was undertaken on the leaf, bark and root extracts of *C. gratissimus* var. *subgratissimus*. The HPLC analysis was undertaken on a Waters 26690 HPLC system operating under the same conditions as described in Chapter 6.5.1.1.

7.5.2 Antimicrobial aspects

Culture, media preparation and assays were undertaken according to the NCCLS (2003) guidelines and methods described by Carson *et al.* (1995) and Eloff (1998a) as described in Chapter 2.5.2. Minimum inhibitory concentrations were determined for the hydrodistilled oil, leaf, bark and root extracts independently on ten test organisms (Figure 7.7). Commercial antimicrobials (ciprofloxacin for bacteria and amphotericin B for yeasts) were included as positive controls in all MIC repetitions and replications.

Synergy / antagonistic interaction: Once the independent MIC was determined on the hydrodistilled oil and leaf, bark and root extracts, the interaction between plant parts was

investigated in two ways. Firstly, stock solutions (64 mg/mL) of each individual plant part extract were prepared and mixed together in the following combinations:

- 1:1 leaf and root
- 1:1 bark and root
- 1:1 leaf and bark
- 1:1:1 leaf, bark and root.

An MIC was determined for these combinations to evaluate if there was a possibility that synergistic interactions were at play. The fractional inhibitory concentration (FIC) was calculated for the leaf with root, bark with root and leaf with bark combination. The FIC is expressed as the interaction of two agents where the concentration of each test agent in combination is expressed as a fraction of the concentration that would produce the same effect when used independently (Berenbaum, 1978). The FIC is then calculated for each test sample independently as specified in the following equations;

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

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

$^{(*a)}$ and $^{(*b)}$ = in this study represents either leaf, bark or root.

The sum of the FIC, known as the FIC index is thus calculated as:

$$\sum FIC = FIC^{(*a)} + FIC^{(*b)}$$

The FIC index (Schelz *et al.*, 2006), is determined as the correlation between the two combined test substances and may be classified as either synergistic (≤ 0.5), additive ($>0.5 – 1$), indifferent ($>1 – 4$) or antagonistic (≥ 4).

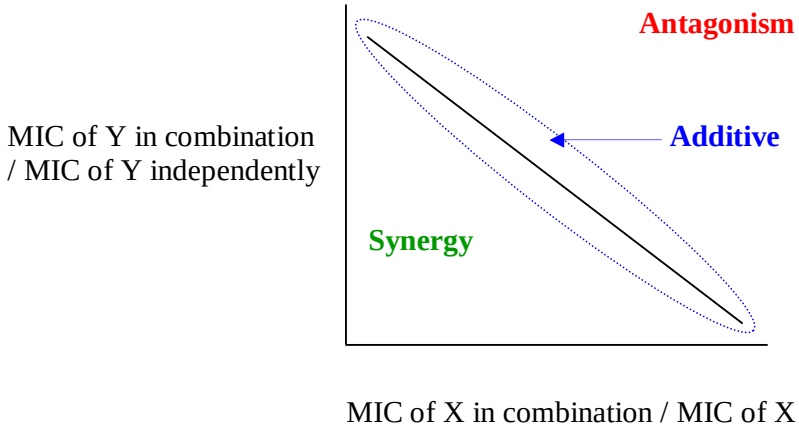


Figure 7.4 Isobologram depicting antagonism, synergy and an additive effect where the relative ratio of Y is plotted to the relative ratio of X.

The second part of this study involved combining the roots and leaves of *C. gratissimus* var. *subgratissimus* in nine ratios i.e. 90:10%; 80:20%; 70:30%; 60:40%; 50:50%; 40:60%; 30:70%; 20:80% and 10:90%. The MIC was determined for all ratios and extracts independently. The study was undertaken in triplicate and the mean MIC values (mg/mL) were plotted on an isobologram using Graphpad Prism® software, allowing for a graphical representation of the interaction of the various combinations. The isobologram can be interpreted by examining the data points of the ratios where the MIC for each concentration is determined in relation to the independent MIC's (shown as a straight line), and extrapolating synergy (below the line), antagonism (above the line) and additive in the vicinity closest to or on the line (Figure 7.4). Conventional antimicrobials (not shown on isobologram) were included in all repetitions. Only values within the 0-1.25 range were plotted on the isobologram.

7.6 Results and discussion

7.6.1 Essential oil chemistry

Ninety-four compounds were identified in the *C. gratissimus* var. *subgratissimus* essential oil representing 93.3% of the total composition (Table 7.2). The toxicity of the plant may possibly be attributed to the high concentrations of the cyclic monoterpene α-phellandrene (20.7%) which acts as a skin irritant and may cause vomiting and diarrhoea if ingested (Windholz, 1976). The presence of α-phellandrene is not restricted to the *gratissimus* species and can be found in other *Croton* species such as *C. stellulifer* as a major constituent (Martins *et al*., 2000) and *C. sakamaliensis* as a minor constituent (Radulović *et al*., 2006).

Table 7.2 Essential oil composition of *C. gratissimus* var. *subgratissimus*.

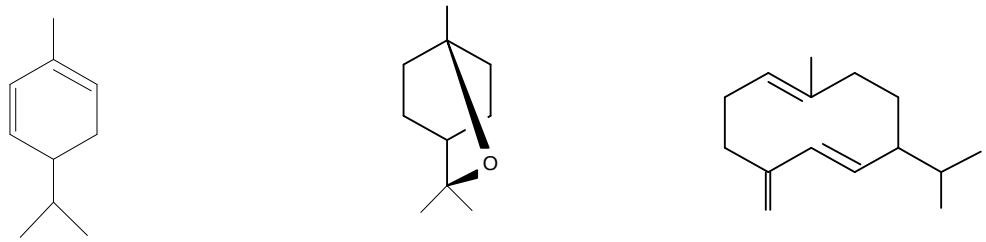
RRI*	Compound name	%
1032	α-pinene	6.7
1035	α-thujene	1.7
1076	camphene	1.8
1118	β-pinene	1.5
1176	**α-phellandrene**	**20.7**
1188	α-terpinene	0.7
1203	limonene	2.7
1213	**1,8-cineole**	**8.3**
1218	β–phellandrene	1.2
1246	(*Z*)-β-ocimene	2.2
1255	γ-terpinene	1.3
1266	(*E*)-β-ocimene	4.5
1280	*p*-cymene	2.8
1290	terpinolene	1.1
1296	methyl heptanoate	tr
1386	1-octenyl acetate	0.2
1391	methyl octanoate	tr
1402	(*Z*)-2-hexen-1-ol	0.1
1450	*trans*-linalool oxide (*Furanoid*)	tr
1453	1-octen-3-ol	2.1
1466	α-cubebene	0.1
1476	*cis*-linalool oxide (*Furanoid*)	0.1
1479	δ-elemene	0.1
1482	(*Z*)-3-hexenyl-2-methylbutyrate	0.1
1493	α-ylangene	0.1
1497	α-copaene	1.6
1532	camphor	5.0
1535	β-bourbonene	0.2
1544	α-gurjunene	tr
1553	linalool	2.6
1554	methyl nonanoate	0.1
1571	*trans-p*-menth-2-en-1-ol	0.1
1589	β-ylangene	0.1
1597	bornyl acetate	0.1
1601	β-copaene	0.1
1611	terpinen-4-ol	0.9
1612	β-caryophyllene	2.1
1617	selina-5,11-diene	0.1
1638	*cis*-p-menth-2-en-1-ol	0.1
1661	alloaromadendrene	0.5
1677	epi-zonarene	0.1
1682	δ-terpineol	0.1
1687	α-humulene	0.4
1689	γ-gurjunene	tr
1706	α-terpineol	1.7

RRI*	Compound name	%
1719	borneol	0.9
1725	verbenone	0.1
1726	**germacrene D**	**8.6**
1730	bicyclosesquiphellandrene	0.1
1739	α-muurolene	0.3
1744	α-selinene	0.1
1755	bicyclogermacrene	0.5
1759	α-cuprenene	0.1
1773	δ-cadinene	0.8
1776	γ-cadinene	0.3
1808	nerol	0.1
1823	p-mentha-1(7),5-dien-2.ol	0.1
1830	2,6-dimethyl-3(*E*),5(*E*),7-octatrien-2-ol	0.1
1854	germacrene B	0.2
1864	*p*-cymen-8-ol	tr
1918	epicubebol	0.1
1941	α-calacorene	tr
1942	benzyl cyanide	tr
1943	palustrol	tr
1957	cubebol	0.2
1984	γ-calacorene	tr
2008	caryophyllene oxide	0.2
2017	methyl eugenol	0.4
2037	salvial-4(14)-en-1-one	0.1
2057	ledol	0.2
2064	α-copaen-11-ol	tr
2069	germacrene D 4-ol	0.1
2080	cubenol	0.1
2088	1-*epi*-cubenol	0.1
2096	elemol	0.3
2101	germacrene D 1,10-epoxide	tr
2127	10-*epi*-γ–eudesmol	tr
2128	salviadienol	tr
2146	spathulenol	0.4
2164	6-*epi*-cubenol	tr
2184	eugenol	0.2
2185	T-cadinol	0.2
2209	T-muurolol	0.2
2214	torreyol	0.1
2255	carvacrol	tr
2256	α-eudesmol	0.2
2257	torilenol	0.1
2258	β-eudesmol	0.6
2264	intermedeol	0.3
2272	alismol	tr
2273	selin-11-en-4α-ol	tr
2369	(2*E*,6*E*)-farnesol	0.7
2389	eudesma-4(15),7-dien-1β-ol	0.1

RRI*	Compound name	%
2622	phytol	0.1
	TOTAL	**93.3**

*****RRI: Relative retention indices calculated against n-alkanes
% calculated from FID data
tr = Trace (< 0.1 %)

Major compounds: Major compounds (Figure 7.5) in the essential oil of *C. gratissimus* var. *subgratissimus* comprise of α-phellandrene (20.7%) and to a lesser extent germacrene D (8.6%) and 1,8-cineole (8.3%), representing 37.6% accumulatively.



α-phellandrene 1,8-cineole germacrene D

Figure 7.5 Chemical structures for major compounds identified in the essential oil of *C. gratissimus* var. *subgratissimus*.

7.6.2 The HPLC analysis of the leaf, bark and root extract

The yield for the root, leaf and bark extracts were 4.34%, 4.04% and 6.41% w/v respectively. The HPLC chromatograms for the root, bark and leaf extracts indicate some similarity (Figure 7.6) and the retention times, UV absorbance maxima and percentage areas of the major peaks (>4.00%), identified in each extract, are summarized in Table 7.3. Two major compounds (indicated in bold, Table 7.3) were present in all three samples (leaf, bark and root) with two major constituents present in both leaf and bark only. Six major compounds were noted in the root material with one compound showing similarity with the bark extract. Further quantitative and qualitative similarities were noted with the minor compounds where analysis indicates similarities between bark and root for five compounds. Similarities between eight minor compounds were also noted between bark and leaf. The leaf and root had only two common compounds. Only three compounds present at low concentrations (0.15-2.25%) were present in all samples. The leaves contained 14 compounds not detected in the other samples. Similarly, the bark exhibited six compounds and root eight compounds, not detected in other parts of the plant.

By combining leaf and root extracts, the number of compounds increases from 14 (leaf) or 8 (root), observed independently, to 22 compounds in combination. As the number of compounds is increased when leaf and root are mixed it is likely that there would be an increased possibility of synergistic interactions that may occur to enhance activity.

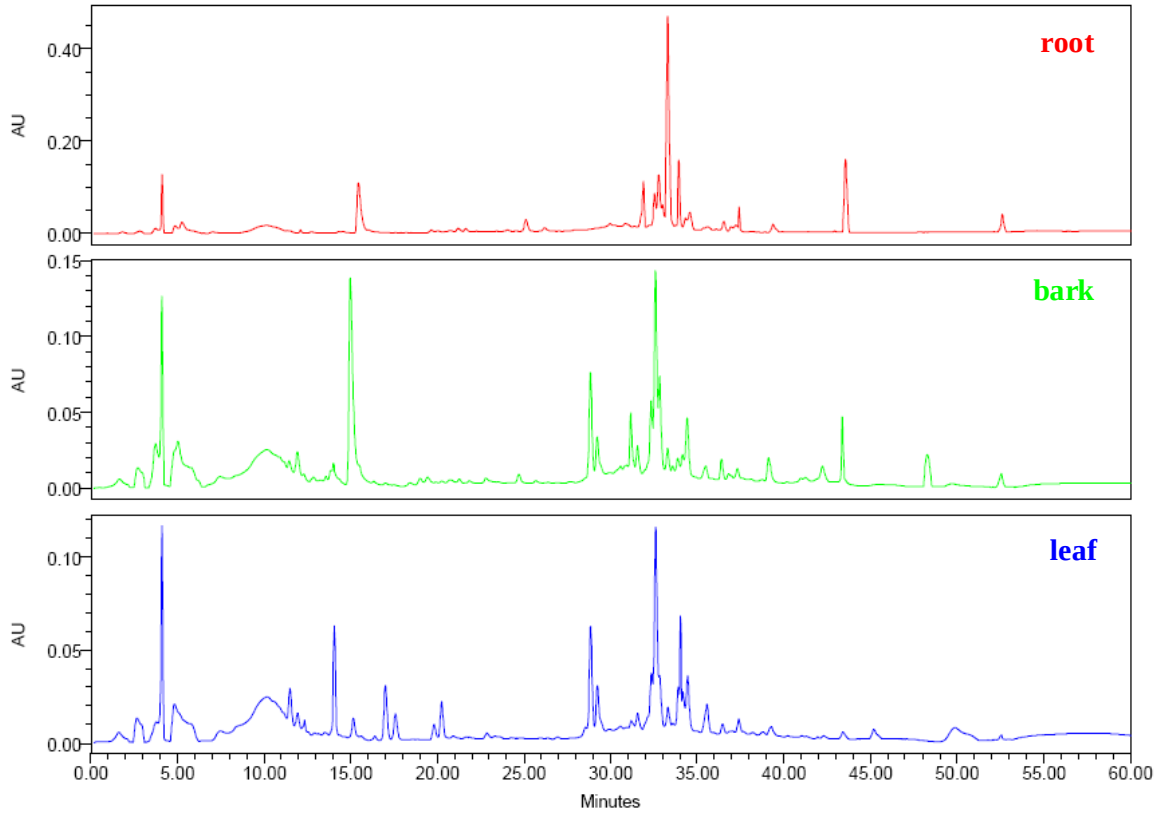


Figure 7.6 The *C. gratissimus* var. *subgratissimus* HPLC chromatogram for root (—), bark (—) and leaf (—).

Table 7.3 The HPLC quantification of leaf, bark and root compounds of *C. gratissimus* var. *subgratissimus*.

Retention time	UV absorbance maxima	Content and area%		
		leaf	bark	root
1.625	201.7;281.7;376.7	0.65	-	0.23
1.652	204.0;279.3	-	0.94	-
2.667	205.2;376.7;598.5	2.18	1,68	0.43
4.113	205.2;375.5;592.2	**6.01**	**4.88**	3.43

Retention time	UV absorbance maxima	Content and area%		
		leaf	bark	root
4.833	205.2;376.7	3.54	-	-
5.032	256.9;592.2	-	3.90	-
5.272	264.0;588.4	-	-	2.88
7.460	202.9;273.4;354.2	1.25	1.32	-
10.164	208.7;273.4;326.8	**19.58**	**13.87**	**6.51**
11.451	208.7;273.4;354.2	2.94	1.42	-
11.925	218.1;260.4;291.2	1.63	1.92	-
14.056	204.0;279.3;376.7	**4.64**	1.19	-
14.981	224.0;267.5;301.9	-	**11.94**	**7.23**
15.153	234.5;324.4	1.23	-	-
16.397	255.7;347.1	0.16	-	-
17.007	228.6;269.9;348.3	2.40	-	-
17.585	231.0;255.7;348.3	1.23	-	-
19.659	234.5;280.5;309.0	-	-	0.37
19.818	231.0;268.7;337.5	0.69	-	-
20.252	229.8;269.9;337.5	1.58	-	-
24.710	242.8;279.3;373.1	-	0.39	-
25.114	233.3;291	-	-	1.44
26.197	243.9	-	-	0.46
28.556	245.1;373.1	0.44	-	-
28.847	236.9;266.3;314.9	**4.80**	**4.56**	-
29.238	266.3;313.7;434.7	2.78	2.58	-
30.934	245.1;265.2;469.5	-	0.94	**4.64**
31.169	266.3;312.5;452.5	0.81	2.58	-
31.368	242.8;565.4	-	-	0.71
31.555	251.0;434.4;595.8	-	1.58	-
32.359	247.5;373.1	2.95	-	0.60
32.539	248.6;399.5;382.4	-	3.60	3.61
32.604	252.2	**9.76**	**7.43**	**5.83**
32.830	241.6;373.1;434.4	1.05	3.70	-
33.310	249.8;373.1;403.1	1.25	1.42	-
33.583	251.0;373.1;434.4	-	0.57	0.31
33.916	254.5;350.6	1.40	-	-
33.935	399.5;565.4	-	-	**4.88**
34.037	249.8;348.3	2.47	-	-
34.174	246.3;334.0;373.1	1.27	0.88	2.25
34.421	243.9;373.1;457.5	-	3.00	-
35.491	245.1;373.1;430.8	0.37	0.81	0.15
35.565	243.9;374.3	1.50	-	-
36.409	242.8;373.1;428.4	-	0.72	1.03
37.235	266.3;565.4	-	-	0.83
37.324	251.0;373.1;399.5	0.48	0.47	-
37.605	249.8;374.3;406.7	-	-	0.07
39.119	251.0;374.3;399.5	0.58	-	-
39.374	245.1;565.4	-	1.14	1.09
43.374	249.8;273.4;374.3	0.39	1.64	**7.65**
45.197	268.7;331.6	0.66	-	-

Retention time	UV absorbance maxima	Content and area%		
		leaf	bark	root
48.280	256.9;273.4;374.3	-	1.82	-
49.857	254.5;319.7;376.7	1.27	-	-
52.610	255.7;582.4	-	-	1.82
	TOTAL	**33**	**29**	**24**

7.6.3 Antimicrobial activity

As noted in literature (Janssen *et al*., 1987; Hinou *et al*., 1989; Yousef and Tawil, 1980; Pattnaik *et al*., 1997; Hammer *et al*., 1999; van Vuuren *et al*., 2006) and in a number of Chapters in this thesis (i.e. *O. asteriscoides*, Chapter 3; *L. javanica*, Chapter 5 and *H. cymosum* subsp. *cymosum*, Chapter 6) the correlation between disc diffusion and MIC assays are not always congruent. As MIC determination demonstrates a more quantitative assay for assessing susceptibility, it was selected as the screening method to determine microbiological susceptibility towards the hydrodistilled oil, leaf, bark and root extracts of *C. gratissimus* var. *subgratissimus* prior to the combination studies. Figure 7.7 represents the antimicrobial activity determined by the MIC assay (mg/mL) of *C. gratissimus* var. *subgratissimus* essential oil, and leaf, bark and root extracts. A literature search on *Croton* species indicated that a number of species had positive antimicrobial activity for their essential oils (Martins *et al*., 2000; Alviano *et al*., 2005). In this study, the essential oils showed moderate activity (4-11 mg/mL) against six of the pathogens studied. Four pathogens (*S. aureus*, *S. epidermidis*, *B. subtilis* and *E. coli*) showed poor susceptibilities (13-32 mg/mL) towards the essential oils.

The leaf, bark and root extract, however, showed higher efficacies than the oils against all the pathogens studied. Upon further analysis of the extracts, no general trend could be found for the Gram-positive organisms. All three Gram-negative organisms (*E. coli, P. aeruginosa* and *K. pneumoniae*) showed highest efficacy for the leaf extracts with the bark and root having equivalent susceptibility patterns. Both yeasts indicated a consistent MIC value for all three extracts i.e. *C. albicans* (6 mg/mL) and *C. neoformans* (2 mg/mL). In a study by Shale *et al.* (1999), information was provided by the traditional healers of Lesotho on the administration of plant material. Healers indicated that the roots were the most commonly used part of the plant. From the leaf, bark and root efficacies noted in this study, the root was found to have either equivalent activities (for six pathogens) or higher efficacies (for four pathogens) of all pathogens studied.

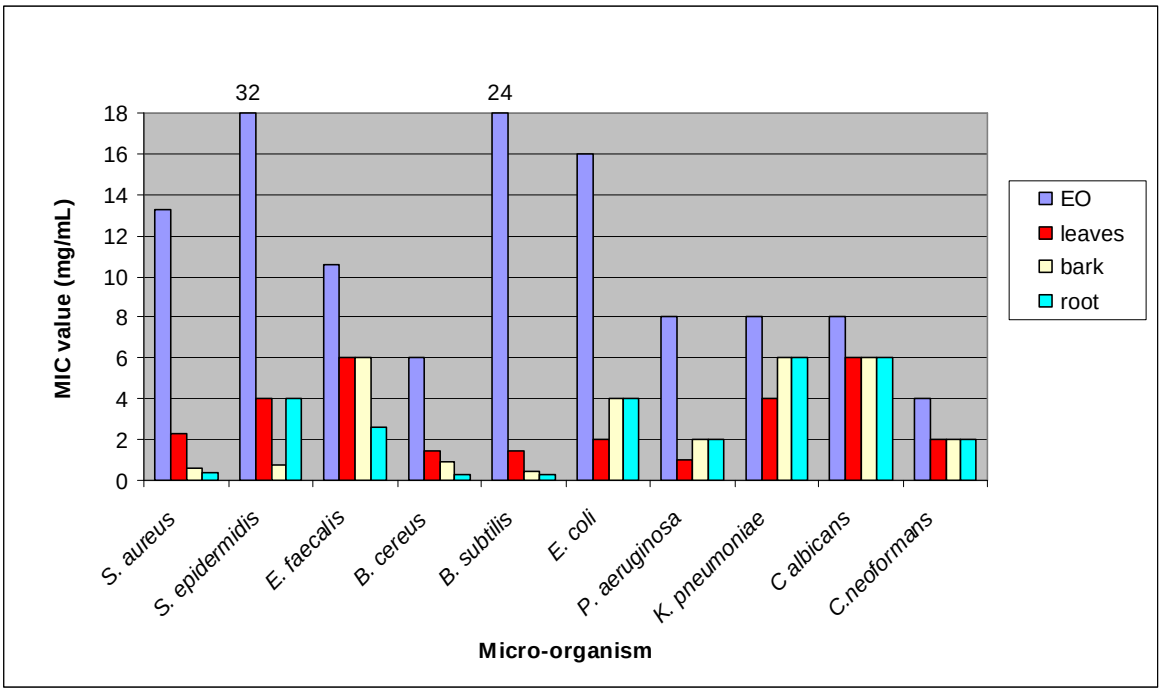


Figure 7.7 The MIC (mg/mL) of *C. gratissimus* var. *subgratissimus* essential oil (EO), and leaf, bark and root extracts.

The traditional use of the root, leaf and bark extracts (van Wyk *et al.*, 1997; Hutchings *et al.*, 1996; von Koenen, 2001) together with the findings that the extracts indicated higher antimicrobial properties than the oils, led the focus of this study towards examination of the interaction between the plant parts when used in combination therapy. The leaf, bark and root of *C. gratissimus* var. *subgratissimus* were mixed in equal aliquots and thereafter a MIC assay carried out on the combinations. Results (Table 7.4) indicate that when a combination of leaf, bark and root is studied, antimicrobial efficacy is mostly enhanced for all pathogens except one (*S. epidermidis*) where the MIC in combination is lower than the leaf and root independently, but higher than the MIC obtained for the bark extract alone. The antimicrobial activity for all other pathogens indicated a lower than or equal to MIC value in combination, than when the leaf, bark and root extracts were investigated independently.

When the leaf and bark extracts were combined activity was enhanced only for *E. faecalis* and *C. albicans.* Antagonism (where the FIC = >4) was found against three of the ten pathogens studied. A predominantly indifferent interaction was noted for leaf and bark combination and only one test organism (*C. albicans*) demonstrated an additive profile.

Table 7.4 The MIC values (mg/mL) for the leaf, root and bark *C. gratissimus* var. *subgratissimus* extracts, singularly and in combination with the FIC[*1].

Pathogen	Leaf	Bark	Root	1:1:1 Leaf, bark and root	1:1 Leaf and bark	1:1 Bark and root	1:1 Leaf and root	Control[*2]
Staphylococcus aureus ATCC 12600	2.3	0.6	0.4	0.3	2.0 (4.2)	0.4 (1.6)	1.0 (2.9)	1.0×10^{-3}
Enterococcus faecalis ATCC 29212	6.0	6.0	2.6	1.3	4.0 (1.4)	1.3 (0.7)	1.5 (0.8)	0.3×10^{-3}
Bacillus cereus ATCC 11778	1.5	0.9	0.8	0.3	1.0 (1.8)	0.3 (0.7)	0.7 (1.3)	0.5×10^{-3}
Staphylococcus epidermidis ATCC 2223	4.0	0.8	4.0	1.0	4.0 (6.0)	3.0 (4.5)	2.0 (1.0)	1.6×10^{-3}
Bacillus subtilis ATCC 6051	1.5	0.5	0.3	0.2	2 .0 (5.3)	0.2 (1.0)	0.5 (1.9)	0.3×10^{-3}
Escherichia coli ATCC 11775	2.0	4.0	4.0	2.0	3.0 (2.3)	1.5 (0.8)	4 .0 (3.0)	0.1×10^{-3}
Klebsiella pneumoniae ATCC 13883	4.0	6.0	6.0	2.0	4.0 (1.7)	3.0 (1.0)	2.0 (0.8)	0.2×10^{-3}
Pseudomonas aeruginosa ATCC 9027	1.0	2.0	2.0	0.6	2.0 (3.0)	1.5 (1.5)	0.5 (0.8)	0.3×10^{-3}
Candida albicans ATCC 10231	6.0	6.0	5.3	1.0	4.0 (0.7)	>8.0 (>8.0)	2.0 (0.8)	1.3×10^{-3}
Cryptococcus neoformans ATCC 90112	2.0	2.0	2.0	0.6	4.0 (2.0)	0.8 (0.8)	0.4 (0.4)	0.6×10^{-3}

FIC[*1] is shown in brackets for all 1:1 combinations where two plants were studied.
[*2] Ciprofloxacin and amphotericin B served as controls for bacteria and yeasts respectively.

For the bark and root combination, a lower MIC value was obtained for pathogens *E. faecalis, B. cereus, B. subtilis, E. coli, K. pneumoniae* and *C. neoformans,* than when investigated against the bark or root independently. The FIC for these pathogens indicated an additive effect (FIC = 0.5-1) when combined in a 1:1 ratio. For *S. aureus* having a FIC of 1.6 and *P. aeruginosa* having a FIC of 1.5, an indifferent profile was observed. The greatest antagonism (FIC = ≥4) was noted for the pathogen *S. epidermidis* (FIC 4.5) and *C. albicans* (FIC >8). *Candida albicans* also showed the least sensitivity when combined, with a MIC value of >8 mg/mL when compared with the root and bark independently having MIC values of 5.3 mg/mL and 6 mg/mL respectively. The FIC for *C. albicans* showed the highest antagonism of all the pathogens studied. No synergy was noted for the root and bark when used in a 1:1 combination.

When the leaf and roots were combined in a 1:1 ratio, the test pathogens *B. cereus, E. faecalis, S. epidermidis, P. aeruginosa, K. pneumoniae, C. albicans* and *C. neoformans* had MIC values lower in combination than when investigated independently. The FIC for these pathogens with

the exception of *B. cereus* and *C. neoformans* showed an additive profile. Only *C. neoformans* demonstrated synergism when combined. The pathogens *S. aureus*, *B. subtilis* and *E. coli* had varying sensitivity patterns in combination with the MIC's, indicating values lower than or equal to one plant part only.

While these results give an indication of interaction between the plant parts, the methodology is restricted to only a 1:1 combination. Isobologram interaction studies were thus undertaken which took into account more than one ratio of the test substance. The leaf and bark combination was selected for further isobologram studies to validate their ethnobotanical therapeutic use.

Table 7.5 Raw data for the isobologram construction for of *B. cereus*.

A.	B.		C.		D.	
Row in plate	Concentration in mg/mL		MIC values		Ratio values	
	X (roots)	Y (leaves)	X (roots)	Y (leaves)	X (roots)	Y (leaves)
1	16.0	0.0	0.80	0.00	1.000	0.000
2	14.4	1.6	0.90	0.10	1.125	0.050
3	12.8	3.2	0.80	0.20	1.000	0.100
4	11.2	4.8	0.70	0.30	0.875	0.150
5	9.6	6.4	0.90	0.60	1.125	0.300
6	**8.0***	**8.0***	**0.25***	**0.25***	**0.313***	**0.125***
7	6.4	9.6	0.20	0.30	0.250	0.150
8	4.8	11.2	0.15	0.35	0.188	0.175
9	3.2	12.8	0.20	0.80	0.250	0.400
10	1.6	14.4	0.20	1.80	0.250	0.900
11	0.0	16.0	0.00	2.00	0.000	1.000

*The 1:1 ratio as determined from the raw data is presented as a red square on the isobolograms.

The isobolograms for all ten pathogens are given with adjacent MIC plots (for relative comparison) in Figures 7.8-7.27. In order to clarify how the isobologram were constructed, a layout of the raw data for one test organism (*B. cereus*) is given in Table 7.5. The first column (A.) represents the microtitre well in which the mixture of the two test substances was prepared. Any arbitrary number may be given. The concentration of the two test substances X and Y were mixed in various ratios. This is reflected in column B. For this study, the roots and leaves were mixed so that the concentration in the first well before further dilution of each ratio is given. Column C presents the MIC results in mg/mL as determined relative to the original concentration given in column B. Column D is the ratio of the MIC results (Column C) with reference to the independent MIC results when not mixed. This ratio is then plotted on the

isobologram which figuratively presents the results as either synergistic, antagonistic or additive (Figure 7.4). Ratios are plotted within the scale 1.25:1.25. Any outlying values with antagonistic profiles greater than that within the scale are not displayed on the isobologram. The raw MIC data can be noted in Table A1, Appendix A.

The isobologram combination studies for the Gram-positive organisms do not show any specific pattern. For *B. cereus*, synergy was observed in four ratio points on the isobolograms where the leaf material were equivalent and in majority (Figure 7.9). However, the FIC (Table 7.4) for *B. cereus* shows indifference (1.3) for the 1:1 ratio of leaf and root combination. Observation of the MIC data for *B. cereus* (Figure 7.8, Table 7.4), indicates that the combined MIC value (0.7 mg/mL) is lower than the MIC value for leaves (1.5 mg/mL) and MIC value for roots (0.8 mg/mL).

The *Staphylococci* having FIC values of 2.9 and 1.0 for the species *aureus* and *epidermidis* respectively (Table 7.4) show similar concentration dependent additive to indifferent profiles, with the 1:1 ratio of the isobole being on the line (Figure 7.11 and 7.13). Results obtained for both combination methods are consistent.

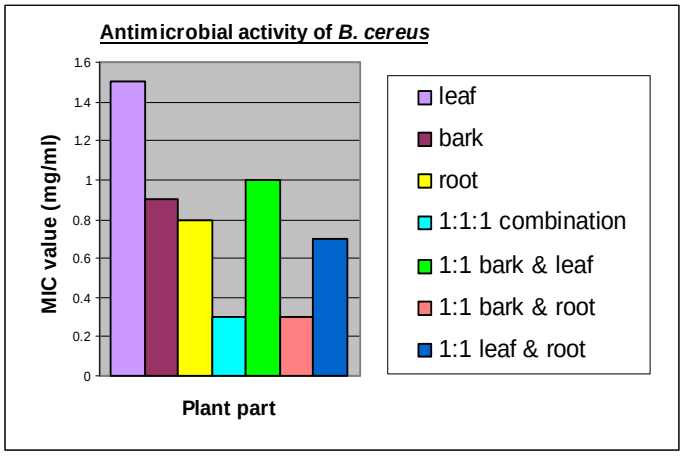


Figure 7.8

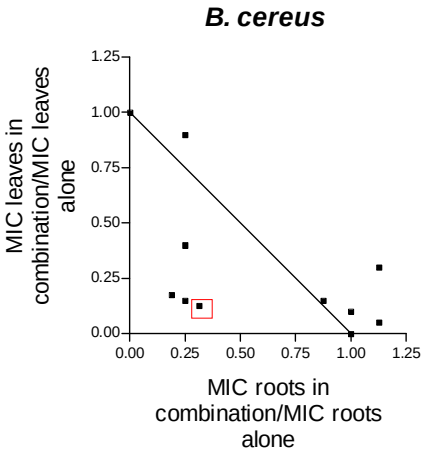


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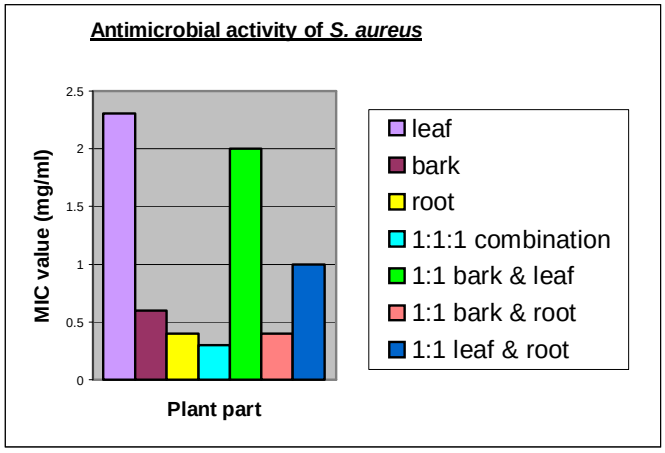


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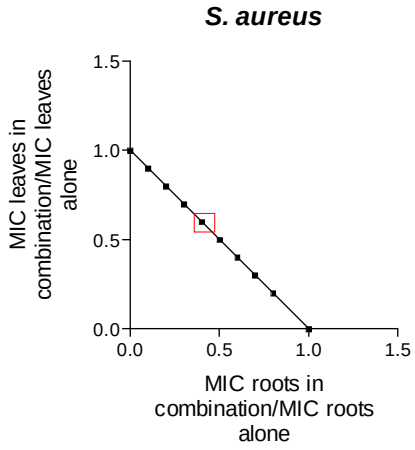


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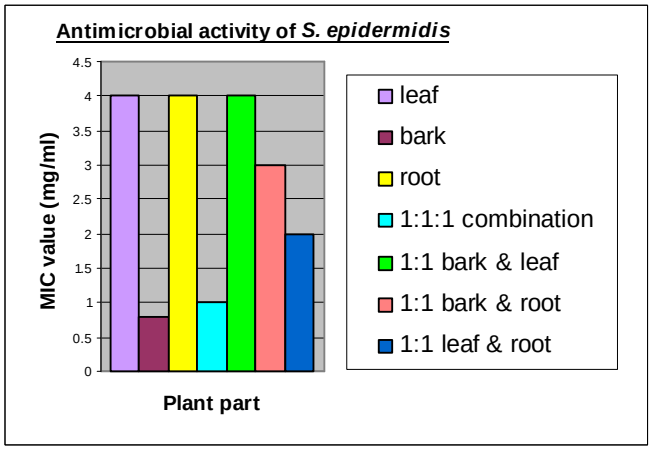


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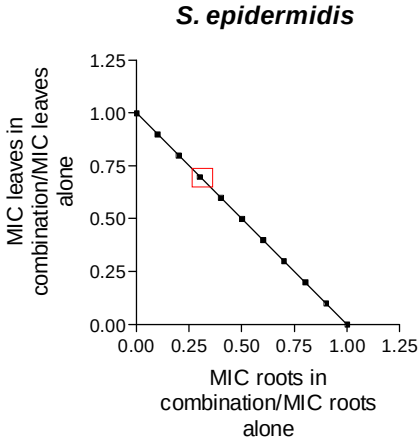


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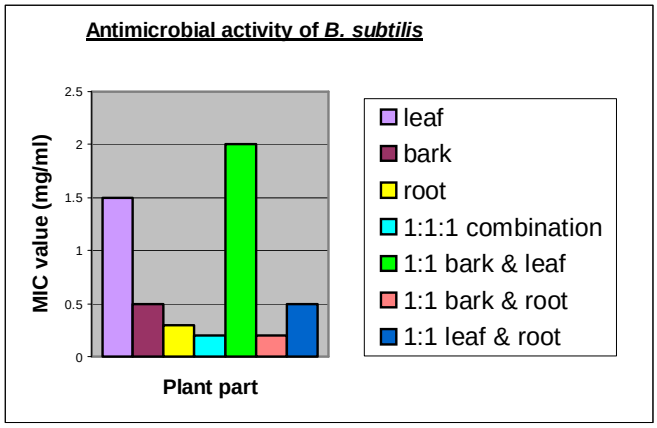


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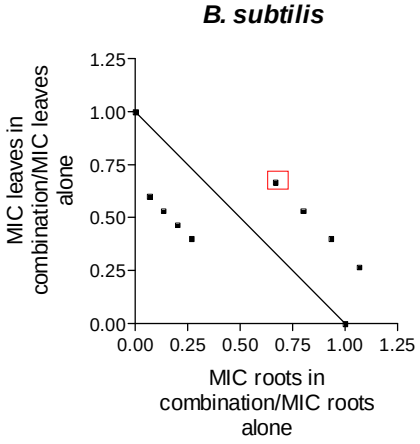


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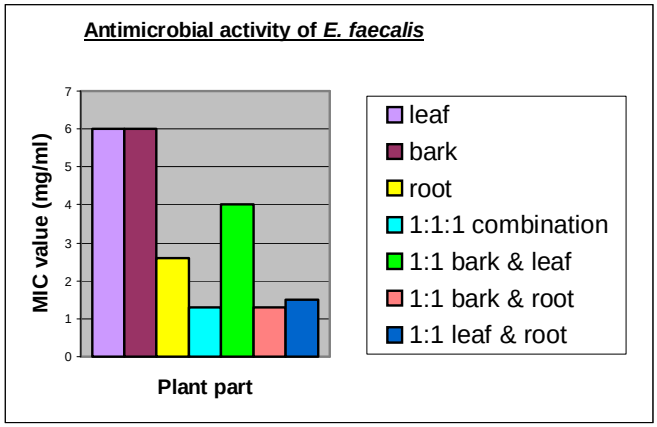


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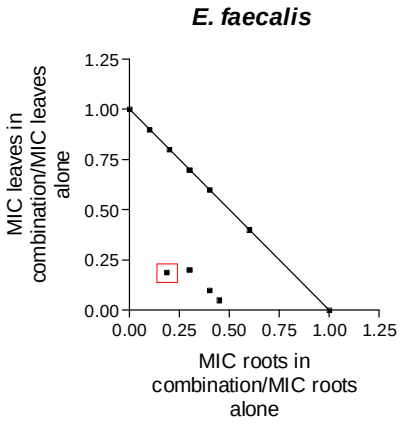


Figure 7.17

Figure 7.8 - Figure 7.17 The antimicrobial efficacy of the Gram-positive test organisms (*B. cereus, S. aureus, S. epidermidis, B. subtilis* and *E. faecalis* respectively) against the leaf, bark and root extract independently and in a 1:1 combination with the adjacent isobologram of leaf and root combination.

Bacillus subtilis indicated antagonism for five of the nine ratios with the highest antagonism noted where the roots were at a 90% concentration. Due to the scale, this is not noted on the isobologram. Where the roots were in majority antagonism was observed. As the root concentration decreases and leaf concentration increases, synergy becomes apparent (Figure 7.15). The isobologram, for this pathogen indicates that depending on the concentration of the combination, synergy may be attained, however, should the concentration vary, antagonism would be encountered. This can also be noted in the adjacent bar graph (Figure 7.14) where the combination is synergistic when comparatively examined with the leaf but not with the root.

For *E. faecalis*, all ratios (Figure 7.17) where leaf concentration was higher than root, a mostly additive profile predominated. Where root concentration is higher than leaf concentration and where concentrations are equal a synergistic profile predominated. The 1:1 ratio (Table 7.4) indicates how efficacy is enhanced when comparatively observing the independent profiles. The FIC (0.8) for *E. faecalis* indicates an additive effect in the 1:1 combination thus corroborating results obtained in the isobologram study.

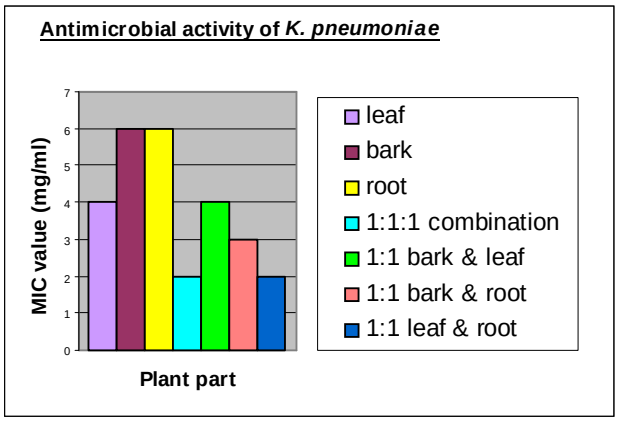


Figure 7.18

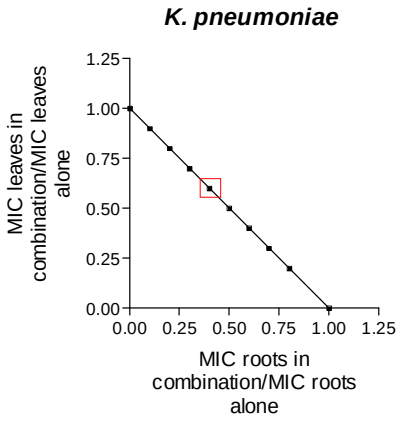


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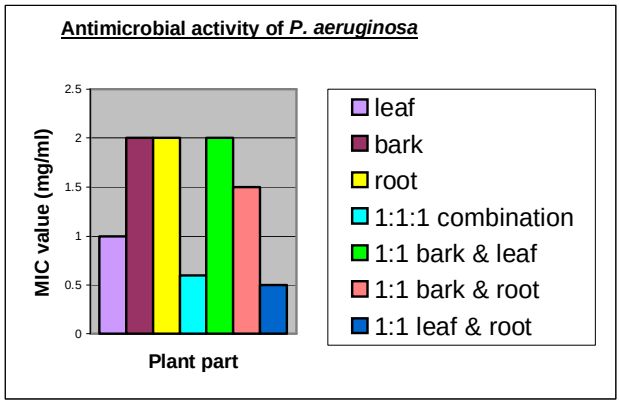


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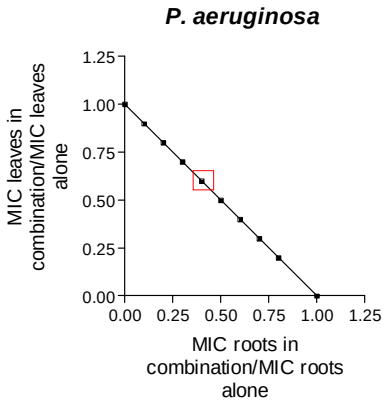


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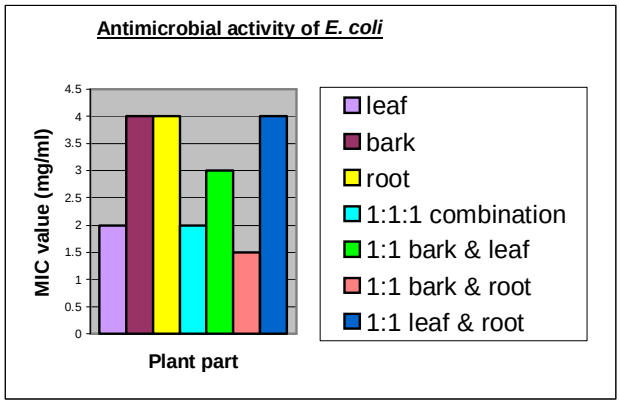


Figure 7.22

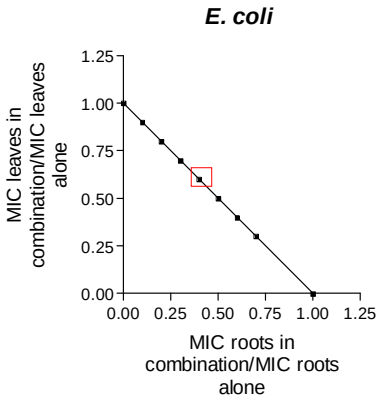


Figure 7.23

Figure 7.18 - Figure 7.23 The antimicrobial efficacy of the Gram-negative test organisms (*K. pneumoniae*, *P. aeruginosa* and *E. coli*) against the leaf, bark and root extract independently and in a 1:1 combination with the adjacent isobologram of leaf and root combination.

The isobolograms for the Gram-negative test organisms: *K. pneumoniae* (Figure 7.19), *P. aeruginosa* (Figure 7.21) and *E. coli* (Figure 7.23) all indicate a ratio dependent pattern. All ratios fall mainly within the additive profile range. The 1:1 combination of leaf and root for *K. pneumoniae* and *P. aeruginosa* indicated an additive effect when the FIC was determined (Table 7.4).

Both yeasts, *C. albicans* and *C. neoformans* investigated in combination studies showed synergism for most ratios when observing the isobologram data (Figure 7.25 and 7.27). The FIC for *C. albicans* (0.8) indicates an additive effect whereas for *C. neoformans* the FIC (0.4) was synergistic. In both 1:1 FIC combination studies and isobologram ratios the leaf / root combination for *C. neoformans* indicate the greatest synergistic patterns. The efficacy against *C. neoformans*, a respiratory pathogen, may be correlated with the traditional use of *C. gratissimus* where the plant is used as a treatment for lung inflammation. The adjacent MIC bar graphs (Figure 7.24 for *C. albicans* and Figure 7.26 for *C. neoformans*) confirm synergism, where it can be noted that MIC values show higher efficacies in combination than when investigated independently.

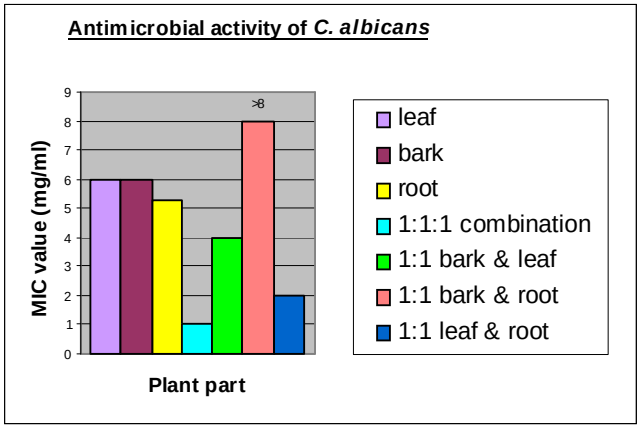


Figure 7.24

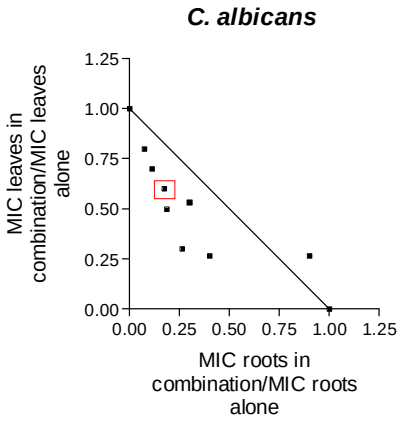


Figure 7.25

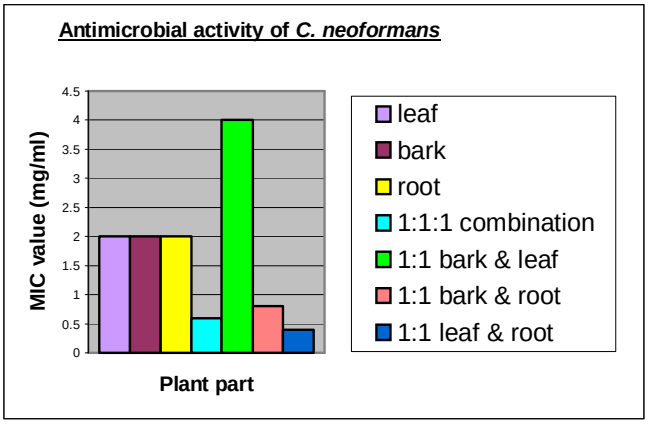


Figure 7.26

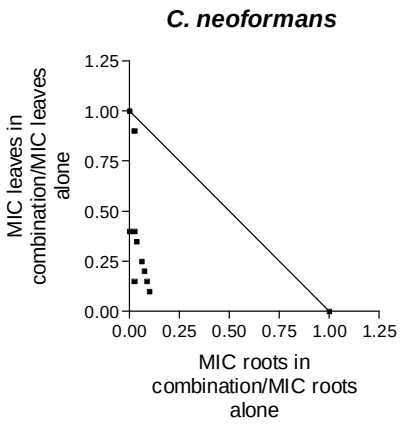


Figure 7.27*

Figure 7.24 - Figure 7.27 The antimicrobial efficacy of the yeasts (*C. albicans* and *C. neoformans*) against the leaf, bark and root extract independently and in a 1:1 combination with the adjacent isobologram of leaf and root combination.

*Due to the close proximity of the ratio points on the isobologram, no red square was inserted for the 1:1 ratio as this would impair data visualization.

Research into antimicrobial synergy configurations has in the past been very vaguely defined and experimental procedures and conclusions based on only one experimental procedure (Lambert *et al*, 2003). Synergy as determined by the FIC method varies in interpretation of results and may either be expressed as an FIC of <1 (Berenbaum, 1978) or ≤ 0.5 (ESCMID, 2000). The FIC synergistic investigation of *C. gratissimus* var. *subgratissimus* was standardized according to the most recent literature (Schelz *et al.*, 2006) with true synergy been expressed as a FIC of ≤ 0.5. Antimicrobial synergy assessment for this study was based on more than one method and as results indicate, methodologies were not always in agreement. In Chapter 11.13, method variation with regard to synergy studies is discussed further. If one compares the FIC methodology with the isobolograms, five of the ten pathogens show congruency, with two additional pathogens (*S. aureus* and *B. subtilis*) showing partial congruency. One possible hypothesis for the variability of these results could be due to dissimilar mechanisms of interaction as noted by different methods (Beale and Sutherland, 1983). It is clear, however, that when comparing the different plant part combinations in a 1:1 ratio, the combination of leaf and root demonstrates the greatest efficacy of the 1:1 combinations, having five additive profiles and one synergistic profile for the ten test pathogens studied. The benefit of combining leaf and root plant parts of *C. gratissimus* var. *subgratissimus* is also apparent in the isobologram studies where a predominant trend is seen where most ratios show an additive profile, or close to an

additive profile, for most pathogens studied. The pathogens showing the highest synergistic profiles (*E. faecalis, C. albicans, C. neoformans* and selectively for *B. cereus*) correlate somewhat with diseases for which the plant is traditionally used for.

Studies such as this give credibility to the use of plant part mixtures for therapeutic treatment. There are many screening studies that focus on the antimicrobial efficacies of different plant parts. Earlier studies (Vlietinck *et al*., 1995) have examined antimicrobial efficacies of the fruit, leaf, root, barks, seeds and whole plant of Rwandese medicinal plants. More recent studies (Eldeen *et al.*, 2005) have examined the leaf, root and bark of ten South African trees for their antimicrobial potential. These studies and many others (Caceres *et al*., 1991; Rabe and van Staden, 1997; Lin *et al*., 1999; Drewes *et al*., 2005) have however, investigated the different parts of the plant as separate entities. A recent study by Lewu *et al.* (2006) comparatively evaluated the antimicrobial efficacy of the roots and leaf material of *Pelargonium sidoides*. Even though the ethnobotanical use of many plants incorporates mixes of the different plant parts, there has been very little scientific evidence to support the interactive efficacies of the different parts of the plant when combined. Furthermore, studies on *Croton* species have narrowly focused on the investigation of specific compounds from the species, such as the isolation of diterpines from the leaves of *Croton zambesicus* (Block *et al.*, 2004), the ethnopharmacological study of the chemical constituents within the roots of *Croton cajucara* (Maciel *et al*., 2000) and investigations on the chemical composition of the bark oil of *Croton stellulifer* (Martins *et al*., 2000). The outcome of this study was to examine the interaction of plant parts rather than taking a reductionist approach to assess the antimicrobial activity of a plant.

7.7 General conclusions

- The major constituents of the essential oil of *Croton gratissimus* var. *subgratissimus* are α-phellandrene (20.7%), germacrene D (8.6%) and 1,8-cineole (8.3%), representing 37.6% accumulatively.

- The extracts of leaf, bark and root when investigated independently indicated better antimicrobial activity (0.3-6.0 mg/mL) than the hydrodistilled leaf essential oil (4.0-32 mg/mL).

- The MIC and FIC results indicated variable efficacies for the plant combinations with the highest efficacy noted for *Cryptococcus neoformans* in the leaf and root combination (MIC value 0.4 mg/mL and FIC of 0.4).

- The pathogens showing the highest synergistic profiles in the isobole method of determining synergy were *Bacillus cereus, Enterococcus faecalis, Candida albicans* and *Cryptococcus neoformans* which correlate with the ethnobotanical uses of the plant to treat respiratory disorders (*Enterococcus faecalis* and *Cryptococcus neoformans*) and abdominal complaints (*Bacillus cereus*).

Chapter 8

A seasonal and geographical study of *Heteropyxis natalensis* Harv. essential oil and effect of stereochemistry on antimicrobial activity.

8.1 Introduction

The family Heteropyxidaceae has only three representatives in southern Africa; *Heteropyxis canescens, Heteropyxis dehniae* and *Heteropyxis natalensis*. The most noteworthy is *Heteropyxis natalensis* Harv., a tree known for its therapeutic properties (Watt and Breyer-Brandwijk, 1962; van Wyk *et al.,* 1997; van Wyk and Gericke, 2000). A literature review on this species identified two seasonal variation studies that had been carried out (Weyerstahl *et al.,* 1992; Chagonda *et al.,* 2000), however, the major essential oil constituents established in these two investigations varied, even though plant sampling was undertaken in the same region. No antimicrobial investigation was undertaken in these two studies. Another study (Gundidza *et al.,* 1993) reported the antimicrobial properties and phytoconstituents of Zimbabwean *H. natalensis* species, but studies were restricted to disc diffusion methods only.

It is known that phytochemicals are often governed by external factors such as soil quality and climate, and biological activity is directly related to the chemical composition of a plant, which may be subject to quantitative and qualitative variation. Seasonal fluctuation of phytoconstituents may or may not impact on the antimicrobial activity. While *H. natalensis* has been cultivated in Zimbabwe for further commercial development (Chagonda *et al.,* 2000), this study on the seasonal variation of South African *H. natalensis* species was undertaken to determine if there was any quantitative and / or qualitative variation in essential oil composition as noted in the Zimbabwean oils. The purpose of this study was to collect, hydrodistil and analyze the essential oils obtained from three individual trees of *H. natalensis* and to characterize the essential oils thereof on a monthly basis. The geographical variation within South Africa was also investigated to determine if oil composition and microbiological activity is dependent on the origin of the plant material.

8.2 Botanical description

Heteropyxis natalensis is a deciduous tree growing to approximately ten metres in height. It has strongly aromatic foliage, a branched trunk and a pale-green bark, which has a distinctive mottled appearance (Figure 8.1). The leaves are small, simple, narrow, oblong / elliptic and are arranged spirally. They are pale-green on the underside with a shiny dark-green upper. The leaves may appear red-tinged when the tree is young (van Wyk *et al.,* 1997). The leaves have a strong lavender odour, which is emitted in the summer; hence the vernacular name "lavender tree". The flowers (Figure 8.2) are yellowish-green, inconspicuous and are in bloom between December and March. They are usually followed by small, oval, dry capsules (fruit), which appear between March and May. The genus name is derived from Greek "Hetero"- meaning different, and a Latin "-pyxis" meaning "the container with the lid". The latter refers to the fruit (capsule), which is sometimes called the pyxidium (Venter and Venter, 2002).



Figure 8.1



Figure 8.2

Heteropyxis natalensis (**Figure 8.1**) and leaves with small inconspicuous flowers (**Figure 8.2**).

8.3 Distribution

Heteropyxis natalensis grows naturally in the north-eastern part of South Africa (Figure 8.3), on the coastal and inland regions extending from the tree's place of origin, which is KwaZulu-Natal, through to Mpumalanga, northern Gauteng regions and Northern Province.

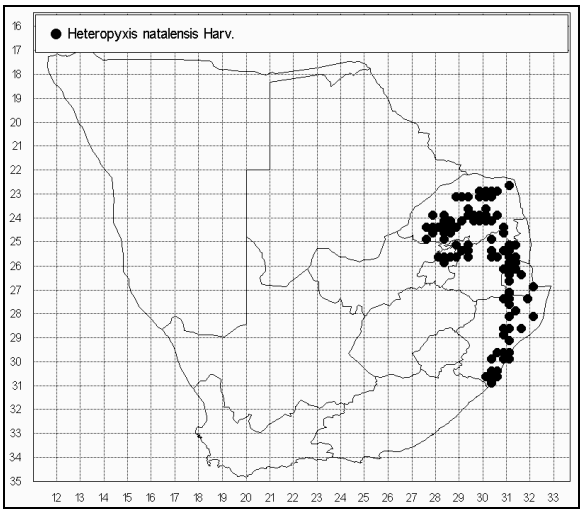


Figure 8.3 The geographical distribution of *H. natalensis* in southern Africa (SANBI).

8.4 Medicinal uses

Heteropyxis natalensis is used traditionally to treat respiratory disorders, as a decongestant and as an anti-infectant (van Wyk *et al.*, 1997). The leaves are mainly used for medicinal purposes and prepared as a tea (van Wyk and Gericke, 2000). A deconcoction of the roots is prepared and the steam inhaled. Both the Venda and Zulu communities have reported on the medicinal values of *H. natalensis* (Watt and Breyer-Brandwijk, 1962).

8.5 Methods

8.5.1 *Chemical aspects*

Plant collection and distillation of essential oils: For the seasonal variation study, the aerial parts from three individual trees (A, B and C) were collected from the Johannesburg Botanical Garden (JHB BG) on a monthly basis, for the year 2004. Due to the semi-deciduous nature of the tree no plant samples were obtained from for June to September and only one tree produced sufficient foliage in October.

Table 8.1 Collection data for *H. natalensis* harvested in the Johannesburg Botanical Garden.

Sample	Voucher	Material distilled (g)	Essential oil yield (% w/w)	Average temperature (ºC)*
A, January	SVV897	1 042.5	0.20	20.5
B, January	SVV898	419.0	0.10	
C, January	SVV899	257.0	0.10	
A, February	SVV922	948.4	0.20	19.5
B, February	SVV923	400.2	0.10	
C, February	SVV924	405.6	0.20	
A, March	SVV954	510.0	0.20	19.0
B, March	SVV955	587.8	0.10	
C, March	SVV956	485.1	0.10	
A, April	SVV972	460.6	0.20	16.0
B, April	SVV973	490.0	0.10	
C, April	SVV974	460.6	0.10	
A, May	SVV993	336.7	0.10	13.0
B, May	SVV994	158.8	0.10	
C, May	SVV995	455.2	0.10	
A, October	SVV1069	450.0	0.10	17.5
A, November	SVV1071	780.0	0.10	18.5
B, November	SVV1072	455.0	0.04	
C, November	SVV1073	816.0	0.20	
A, December	SVV1074	1 296.0	0.10	19.5
B, December	SVV1075	811.0	0.10	
C, December	SVV1076	840.0	0.10	

* Average temperature at sampling is calculated as the mean of the maximum and minimum temperature for that month (http://www.weathersa.co.za/FcastProducts/LongRange/ViewSeasonEyeBall.jsp).

For the geographical variation study, plants were collected from Cullinan and Verena (Gauteng); Nelspruit (Mpumalanga); Lagalametse and Waterberg (Northern Province) and Balakane (Swaziland). With the ex```ception of the Balakane sample (collected in November), all collections were undertaken in the month of February to avoid any impact on possible seasonal variations that may occur.

Voucher specimens for seasonal (Table 8.1) and geographical (Table 8.2) sampling were deposited in the Department of Pharmacy and Pharmacology, University of Witwatersrand. The plants were distilled as described in Chapter 2.5.1.

Table 8.2 Collection data for *H. natalensis* sourced from various localities.

Locality	Voucher	Material distilled (g)	Essential oil yield (% w/w)
Cullinan	AMV771	700.0	0.3
Verena	PMB703	559.0	0.2
Nelspruit	ADC721	421.5	0.2
Lagalametse	ADCAV181	450.0	0.1
Waterberg	PMB738	499.2	0.1
Balakane	ADCAV140	380.0	0.1

Gas chromatography (GC) and GC-MS data: The GC analysis was performed on a Shimadzu 17A gas chromatograph using the parameters as described in Chapter 2.5.1. Oil samples were quantitatively analyzed with GC-MS using the Hewlett-Packard 1800A GCD system (Chapter 2.5.1.). Using quantitative data obtained from the GC-MS analysis, a cluster analysis was performed on the essential oils from three seasonal samples (February, plants A, B and C) and a further six *H. natalensis* samples from different localities. A dendrogram (Figure 8.8) presents the data using the NTSYS-pc software package (Version 2.0) analysed by a phenetic program (Rohlf, 1998). Correlation of similarity was selected for the basis of analysis and the unweighted pair-group method with arithmetic average (UPGMA) was used for cluster definition.

Thin layer chromatography (TLC): TLC plates were developed for the essential oil samples obtained from the seasonal variation study to establish if there was obvious seasonal variation in the essential oil components. Samples were prepared by placing 2 µl of diluted (1:7 hexane, Merck) *H. natalensis* essential oil on silica gel TLC plates (Alugram ® Sil G/UV_{254}, 0.2 mm). The plates were developed with ethyl acetate and toluene (Merck) at a ratio of 7:93 and dried at ambient temperature. Plates were observed under ultraviolet light (254 nm) and thereafter sprayed with an anisaldehyde-acetic-sulphuric acid reagent. This was prepared by mixing 0.5 mL anisaldehyde (Fluka) with 10 mL glacial acetic acid (Merck), followed by 85 mL methanol (Merck). The TLC plates were subsequently placed in the oven at 110 °C for five minutes to enable visualization of the separated compounds (Wagner and Bladt, 1995).

8.5.2 Antimicrobial aspects

Culture, media preparation and assays were undertaken according to the NCCLS (2003) guidelines (Chapter 2.5.2.). Quantitative MIC assays (Carson *et al*., 1995; Eloff, 1998a; NCCLS, 2003) were performed on the hydrodistilled oil to determine if:

- The antimicrobial efficacy supports the traditional use.
- Results correlate with that found in literature (Gundidza *et al*., 1993).
- Seasonal variation exists.
- Geographical variation is evident between distant populations.
- Structure-activity relationships between major constituents and biological activity were evident.

As the traditional use of *H. natalensis* is for respiratory tract infections, eight microbial test organisms were selected for the seasonal variation study based on their respiratory pathogenesis. A clinical Gram-negative respiratory pathogen, *Moraxella catarrhalis* was included in the study. This opportunistic respiratory pathogen has severe implications in upper and lower respiratory tract infections, bronchitis, pneumonia and may even be associated with sinusitis and otitis media (Laurans and Orfila, 1991; Verduin *et al*., 2002). The study on the Zimbabwean *H. natalensis* species undertaken by Gundidza *et al*. (1993) has shown antimicrobial sensitivity towards this pathogen and was included in this study to determine if similar sensitivity patterns exist for the South African *H. natalensis* samples.

In addition to the *H. natalensis* samples examined for seasonal variation, a further six *H. natalensis* samples were selected from different regions to determine any geographical variation within the MIC data. Antimicrobial MIC assays were limited to five pathogens (Table 8.6) due to limited oil acquisition. Pathogen selection was based on the traditional use of *H. natalensis.*

All MIC assays were undertaken at least in triplicate with the seasonal mean and standard deviation presented in Table 8.5. The geographical mean with standard deviation is presented in Table 8.6. The geographical MIC data (Table 8.6) was grouped according to sensitivity, with poor susceptibility classified as 16 - >16 mg/mL, moderate susceptibility with MIC values 8 - <16 mg/mL, moderate to good susceptibility with MIC values 4 - <8 mg/mL and good susceptibility with MIC values <4 mg/mL. A cluster analysis was performed from this data using the same parameters as described with the GS-MS cluster analysis and presented in Figure 8.10.

Interaction between limonene and 1,8-cineole: The GC-MS data confirmed that limonene and 1,8-cineole are predominantly present as the two major compounds in *H. natalensis*. The enantiomeric configuration of limonene with 1,8-cineole was investigated for antimicrobial activity according to the MIC microtitre plate method as described previously. The compounds 1,8-cineole at 98.0% purity (Lot 1054365) was obtained from Sigma-Aldrich. S(-)-Limonene at 99.0% purity (Lot 054076) was obtained from Fluka and (R)-(+)-limonene at 97.0% purity (Lot 301Tl-101) was obtained from Sigma-Aldrich. The compounds were prepared at starting concentrations 128 mg/mL and assayed with the *H. natalensis* essential oil. Assays were undertaken in triplicate and the mean documented in Table 8.7.

Table 8.3 Raw data for the isobologram construction of *S. aureus*.

Concentration in mg/mL		**MIC values**		**Ratio values**	
X (+/- limonene)	**Y (1,8-cineole)**	**X (+/- limonene)**	**Y (1,8-cineole)**	**X (+/- limonene)**	**Y (1,8-cineole)**
32.0	0.0	4.00	0.00	1.000	0.000
28.8	3.2	7.20	0.80	1.800	0.100
25.6	6.4	4.80	1.20	1.200	0.150
22.4	9.6	4.20	1.80	1.050	0.225
19.2	12.8	3.60	2.40	0.900	0.300
16.0*	**16.0***	**3.00***	**3.00***	**0.750***	**0.375***
12.8	19.2	2.40	3.60	0.600	0.450
9.6	22.4	2.40	5.60	0.600	0.700
6.4	25.6	1.60	6.40	0.400	0.800
3.2	28.8	0.80	7.20	0.200	0.900
0.0	32.0	0.00	8.00	0.000	1.000

*The 1:1 ratio as determined from the raw data is presented as a square on the isobolograms.

The optically active monoterpene, limonene was used to determine if microbes respond differently when exposed to the (+), (-) and / or racemic mixture of limonene in combination with 1,8-cineole. The isobologram ratio method as described in Chapter 7 (Berenbaum, 1978; Williamson, 2001) was used and nine ratios i.e. 90:0%; 80:20%; 70:30%; 60:40%; 50:50%; 40:60%; 30:70%; 20:80% and 10:90% of each enantiomeric form and the racemate were thoroughly mixed with 1,8-cineole. The MIC values (mg/mL) were determined for each concentration independently and for the nine ratios, and raw MIC data can be noted in Table A2, Appendix A. The MIC of all relevant ratios (values within the 0-1.25 range) were plotted as points on an isobologram relative to the MIC of limonene and 1,8-cineole independently (shown as a straight line), allowing for a figurative representation of the interaction of the various combinations. Investigations were undertaken in duplicate on the following reference test

organisms: *S. aureus* ATCC 12600, (Gram-positive); *P. aeruginosa* ATCC 9027, (Gram-negative) and a yeast *C. neoformans* ATCC 90112. Conventional antimicrobials (not shown on the isobologram) were included in all repetitions to ensure susceptibility of the test organism. The raw data for the isobologram construction for one test organism (*S. aureus*) is given in Table 8.3.

8.6 Results and discussion

8.6.1 Essential oil chemistry

The GC-MS data identified approximately 173 compounds in the *H. natalensis* essential oil representing 72.0−97.8% of the total composition (Table 8.4).

Major compounds: Two major compounds (limonene and 1,8-cineole) identified by GC were predominantly present in all seasonal samples. Figure 8.7 shows the monthly variation of the different concentrations of the main compounds limonene and 1,8-cineole in relation to the sum of the remainder of the constituents as determined by GC. Three individual trees (samples February A, B and C) were selected for further GC-MS analysis. Major compounds confirmed were 1,8-cineole (23.9-41.2%) and limonene (18.1-25.4%). The *H. natalensis* (geographical study) samples identified limonene and 1,8-cineole as major constituents in four localities. Linalool was found as a major constituent in the samples from Nelspruit (16.7%) and Lagalametse (11.4%). (*Z*)-3-Hexenyl nonanoate (16.0%) was only present in the Lagalametse sample. ß-Pinene at 25.2% was identified as the major constituent in the Balakane sample. (*E*)-Nerolidol was present as a major constituent in the samples JHB BG, sample A (12.5%) and C (16.3%) and only present in negligible quantities in the other geographical samples. (*E*)-Nerolidol, limonene and ß-pinene were not present in the Lagalametse sample. All major compounds detected in the various *H. natalensis* samples are given in Figure 8.4.

Table 8.4 Essential oil composition as determined by GC-MS for *H. natalensis* samples (seasonal and geographical variation study).

RRI*	Compound name	Gauteng					Mpumalanga	Northern Province		Swaziland
		JHB BG (Feb)			Cullinan	Verena	Nelspruit	Lagalametse	Waterberg	Balakane
		A	B	C						
1032	α-pinene	1.7	3.2	3.1	3.0	1.9	2.7	tr	3.1	1.9
1035	α-thujene	0.4	1.2	1.1	0.6	0.6	1.0	-	0.7	tr
1076	camphene	-	-	tr	0.1	-	-	-	-	0.1
1118	β-**pinene**	1.1	2.0	1.9	7.6	3.2	6.2	-	2.5	**25.2**
1132	sabinene	-	0.1	0.4	0.1	0.1	0.6	-	0.1	tr
1146	2-methylbutyl acetate	-	-	-	-	-	-	0.1	-	-
1174	myrcene	0.5	1.8	2.0	1.6	1.2	3.2	-	1.4	5.1
1183	*p*-mentha-1,7(8)-diene (=*Pseudolimonene*)	5.2	7.1	4.6	6.0	3.7	3.0	-	6.5	tr
1188	α-terpinene	-	-	-	0.1	-	-	-	-	-
1203	**limonene**	**18.1**	**25.4**	**16.5**	**22.8**	**19.2**	**15.0**	-	**23.6**	1.8
1213	**1,8-cineole**	**33.6**	**41.2**	**23.9**	**23.5**	**29.4**	**21.7**	0.2	**39.2**	-
1246	(*Z*)-β-ocimene	-	0.3	0.1	0.4	tr	0.2	-	-	0.4
1255	γ-terpinene	-	0.2	-	1.1	-	-	-	-	tr
1265	(*E*)-β-ocimene	-	0.8	-	1.5	tr	0.7	-	-	2.5
1274	2-heptyl acetate	-	-	-	0.1	0.1	0.1	0.2	-	0.2
1275	2-methylbutyl butyrate		0.1	tr	0.3	0.3	0.1	0.1	0.4	tr
1280	*p*-cymene	3.5	2.5	3.3	3.7	4.3	4.2	0.1	5.8	0.4
1290	terpinolene	-	0.1	-	0.2	-	-	-	-	0.1
1303	amyl isovalerate	-	-	-	-	-	-	-	0.1	-
1345	4-pentenyl butyrate†	0.4	0.5	1.2	1.2	tr	0.2	0.4	0.3	0.2
1391	(*Z*)-3-hexenol	-	-	-	-	-	-	-	0.4	tr
1395	2-nonanone	-	-	0.3	0.7	0.3	0.6	2.4	-	1.1
1398	3-methyl-2-butenyl butyrate†	0.3	0.3	1.1	0.6	0.4	0.1	0.4	0.2	0.1
1413	rose furan	-	-	0.2	-	-	0.1	-	-	0.1
1429	perillen	-	-	-	-	tr	0.1	-	-	tr
1450	*trans*-linalool oxide (*Furanoid*)	-	-	-	tr	tr	0.4	8.8	0.1	tr
1458	*cis*-1,2-limonene epoxide	0.3	-	0.1	-	tr	0.1	-	-	-
1466	α-cubebene	-	-	-	-	-	-	-	-	0.1
1468	*trans*-1,2-limonene epoxide	0.2	-	-	-	-	-	-	-	-
1469	3-methyl butyl hexanoate (=*Isoamyl hexanoate*)	-	-	-	-	-	0.1	-	-	tr

RRI*	Compound name	Gauteng					Mpumalanga	Northern Province		Swaziland
		JHB BG (Feb)			Cullinan	Verena	Nelspruit	Lagalametse	Waterberg	Balakane
		A	B	C						
1470	2-nonyl acetate	-	-	-	-	-	-	0.3	-	-
1471	(*Z*)-3-hexenyl butyrate	-	-	0.2	0.1	-	-	-	0.1	-
1473	(*E*)-2-hexenyl butyrate	-	tr	-	-	0.2	-	-	-	-
1474	*trans*-sabinene hydrate	-	-	-	-	-	0.1	-	-	-
1476	(*Z*)-β-ocimene epoxide	-	-	tr	-	-	tr	-	-	tr
1478	*cis*-linalool oxide (*Furanoid*)	-	-	tr	tr	-	0.3	8.0	0.1	tr
1482	fenchyl acetate	0.1	tr	tr	tr	-	-	-	-	tr
1483	octyl acetate	-	-	-	-	-	tr	-	-	tr
1497	α-copaene	-	-	tr	0.1	0.1	-	-	tr	0.1
1498	(*E*)-β-ocimene epoxide	-	-	0.1	-	0.1	0.1	-	-	0.1
1506	decanal	-	-	-	-	-	-	0.1	-	-
1521	2-nonanol	-	-	-	tr	-	-	0.4	-	-
1541	benzaldehyde	-	tr	tr	-	tr	tr	-	0.1	-
1553	**linalool**	0.1	1.2	0.5	8.0	0.2	**16.7**	**11.4**	2.1	1.0
1559	8,9-limonene epoxide-I	tr	-	-	-	-	-	-	-	-
1565	linalyl acetate	-	tr	-	0.1	-	0.1	0.1	tr	-
1571	*trans-p*-menth-2-en-1-ol	0.1	tr	0.1	tr	0.1	0.1	-	-	-
1586	pinocarvone	-	-	tr	-	-	0.1	-	-	0.1
1589	β-ylangene	-	-	-	-	-	-	-	-	0.1
1590	3-methyl-2-butenyl hexanoate[†]	-	-	tr	-	-	-	0.1	-	tr
1591	fenchyl alcohol	-	-	-	-	-	tr	-	-	0.2
1597	bornyl acetate	-	-	-	-	-	-	-	-	0.2
1600	β-elemene	-	-	0.1	-	-	0.1	-	-	0.1
1601	β-copaene	-	-	tr	-	0.1	-	-	-	0.2
1602	6-methyl-3,5-heptadien-2-one	-	-	0.2	-	0.1	-	-	-	-
1605	2-undecanone	-	-	-	0.1	-	0.1	0.8	-	-
1611	terpinen-4-ol	1.4	1.6	1.4	0.7	0.8	2.1	-	2.3	0.1
1612	β-caryophyllene	-	0.1	0.5	0.7	0.1	0.1	-	tr	1.9
1616	hotrienol	-	-	-	-	-	-	0.3	-	-
1625	4,4-dimethyl but-2-enolide	-	-	-	-	-	-	0.1	-	-
1628	aromadendrene	-	-	0.1	-	tr	-	-	-	-
1630	terpinen-4-yl acetate (*=4-Terpinenyl*	-	-	-	-	tr	tr	-	-	-

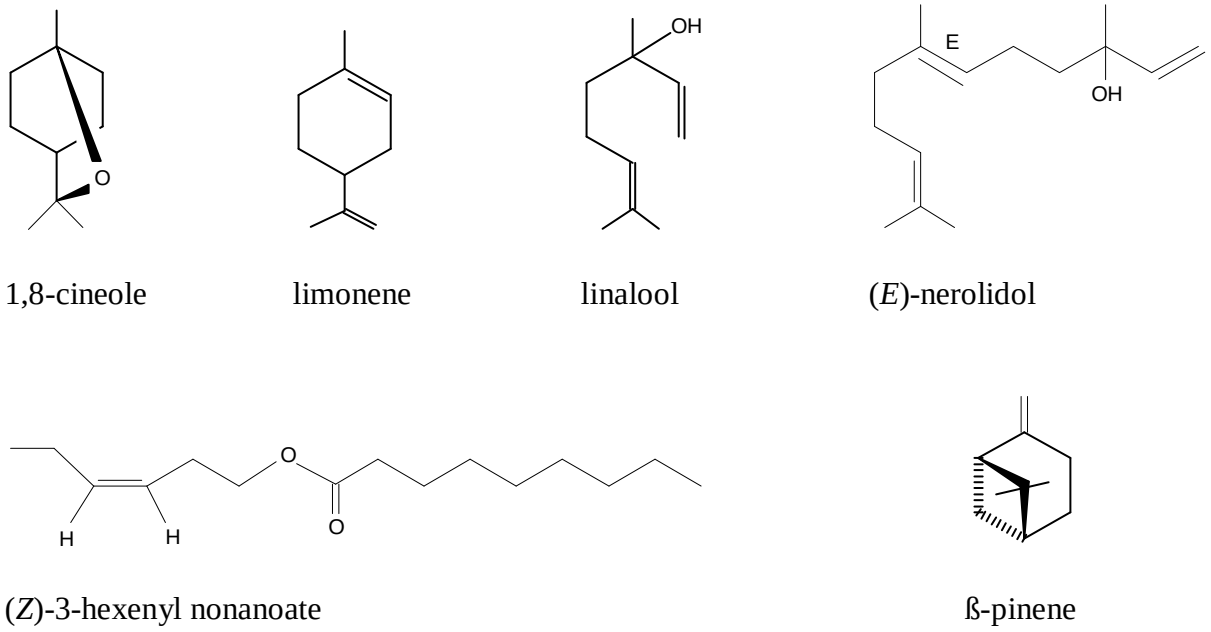
RRI*	Compound name	Gauteng					Mpumalanga	Northern Province		Swaziland
		JHB BG (Feb)			Cullinan	Verena	Nelspruit	Lagalametse	Waterberg	Balakane
		A	B	C						
	acetate)									
1638	*cis-p*-menth-2-en-1-ol	-	-	-	tr	-	0.1	-	-	-
1639	*trans-p*-mentha-2,8-dien-1-ol	0.1	tr	0.1	-	0.2		-	-	-
1641	*cis*-β-terpineol	-	tr	tr	-	tr	0.1	-	-	-
1648	myrtenal	-	-	-	-	-	0.1	-	-	0.3
1661	alloaromadendrene	-	-	-	-	-	-	-	-	0.3
1664	nonanol	-	-	-	-	-	-	0.2	-	-
1670	*trans*-pinocarveol	0.1		0.1	tr	0.2	0.1	-	-	0.4
1671	acetophenone	-	tr	-	-	-	-	-	0.1	-
1678	*cis-p*-mentha-2,8-dien-1-ol	-	-	0.1	-	0.1	-	-	-	-
1681	4-methyl-4-vinyl butyrolactone	-	-	-	-	-	-	5.3	-	-
1682	δ-terpineol	0.5	0.4	0.2	0.1	0.3	0.2	-	0.4	-
1687	α-humulene	0.1	0.2	0.8	0.3	0.3	0.4	-	0.1	0.5
1688	selina-4,11-diene(=4,11-*Eudesmadiene*)	-	-	-	-	-	-	-	-	0.2
1700	*p*-mentha-1,8-dien-4-ol (=*Limonen-4-ol*)	-	tr	tr	-	0.1	-	-	0.1	-
1704	myrtenyl acetate	-	-	-	-	-	tr	-	-	-
1705	γ-muurolene	-	-	-	-	-	-	-	-	0.3
1706	α-terpineol	3.1	3.2	2.3	1.0	3.5	3.0	-	2.8	2.5
1707	δ-selinene	-	-	-	-	-	-	-	-	0.1
1709	α-terpinyl acetate	0.1	tr	-	0.1	0.1	0.1	-	0.1	-
1718	4,6-guaiadiene (=γ-*Guaiene*)	-	-	-	-	-	-	-	-	tr
1719	borneol	-	-	-	-	-	-	-	-	0.2
1723	*cis*-1,2-epoxy-terpin-4-ol	0.2	tr	0.1	-	0.1	0.1	-	tr	tr
1733	neryl acetate	-	-	-	-	0.1	0.1	-	-	-
1739	α-muurolene	-	0.1	0.2	-	0.3	0.1	-	-	1.0
1740	valencene	-	-	0.1	-	0.1	-	-	-	0.2
1742	β-selinene	-	-	-	0.1	-	0.2	-	-	-
1744	α-selinene	-	tr	0.1	0.1	0.1	0.1	-	-	0.6
1750	*cis*-linalool oxide (*Pyranoid*)	-	-	-	-	-	-	1.9	-	-
1751	carvone	0.5	0.1	0.2	-	0.4	tr	-	0.2	-
1758	*cis*-piperitol	-	tr	tr	-	tr	tr	-	-	-
1759	(*E,E*)-α-farnesene	-	-	-	-	-	-	-	-	tr

RRI*	Compound name	Gauteng					Mpumalanga	Northern Province		Swaziland
		JHB BG (Feb)			Cullinan	Verena	Nelspruit	Lagalametse	Waterberg	Balakane
		A	B	C						
1765	geranyl acetate	-	tr	-	0.1	0.2	0.1	0.2	0.1	0.1
1770	*trans*-linalool oxide (*Pyranoid*)	-	-	-	-	-	tr	2.0	-	-
1773	δ-cadinene	0.1	0.1	0.1	0.1	0.1	0.1	-	0.1	1.2
1776	γ-cadinene	-	0.1	0.2	0.1	0.4	0.1	-	-	0.6
1797	*p*-methyl acetophenone	-	-	-	-	-	tr	-	-	-
1798	methyl salicylate	-	-	-	tr	-	tr	-	-	-
1804	myrtenol	0.1	-	-	-	0.2	0.1	-	-	0.3
1807	α-cadinene	-	-	-	tr	tr	tr	-	-	-
1808	nerol	-	-	-	-	-	0.1	-	-	-
1811	*trans-p*-mentha-1(7),8-dien-2-ol (= *trans-2-hydroxy pseudolimonene*)	-	tr	-	-	-	-	-	-	-
1826	γ-heptalactone	-	-	-	-	-	-	1.1	-	-
1830	2,6-dimethyl-3(*E*),5(*E*),7-octatriene-2-ol	-	tr	-	tr	-	0.1	-	-	-
1845	*trans*-carveol	0.5	0.1	0.3	tr	0.5	0.1	-	0.2	-
1853	*cis*-calamenene	0.1	tr	0.1	-	0.2	0.1	-	0.1	0.6
1857	geraniol	-	-	-	0.1	-	0.3	-	-	-
1864	*p*-cymen-8-ol	0.2	tr	0.1	-	0.2	0.2	-	0.1	0.1
1875	*trans*-2-hydroxy-1,8-cineole	0.1	-	-	-	-	-	-	-	-
1882	*cis*-carveol	0.2		0.1	-	-	tr	-	0.1	-
1883	benzyl butanoate	0.1	0.1	0.3	0.1	0.2	tr	0.2	0.1	-
1896	*cis-p*-mentha-1(7),8-dien-2-ol (= *cis-2-hydroxy pseudolimonene*)	-	tr	-	-	-	-	-	tr	-
1901	geranyl butyrate	-	tr	0.1	0.1	0.2		-	tr	-
1941	α-calacorene	-	tr	tr	tr	-	tr	-	-	0.1
1949	**(*Z*)-3-hexenyl nonanoate**	-	-	-	-	-	-	**16.0**	-	-
1969	*cis*-jasmone	-	-	-	0.1	0.1	tr	-	tr	0.1
1989	2,6,10-trimethyl-7,10-epoxy-2,11-dodecadien-6-ol (=*Nerolidol oxide*)	0.4	-	-	-	-	-	-	-	-
2001	isocaryophyllene oxide	-	-	0.2	-	0.5	0.2	-	tr	0.1
2008	caryophyllene oxide	1.3	1.2	2.2	4.2	7.2	1.5	4.6	1.3	4.5
2016	2,6,10-trimethyl-7,10-epoxy-2,11-dodecadien-6-ol isomer (=*Nerolidol oxide*	0.6	-	-	-	-	-	-	-	-

RRI*	Compound name	Gauteng					Mpumalanga	Northern Province		Swaziland
		JHB BG (Feb)			Cullinan	Verena	Nelspruit	Lagalametse	Waterberg	Balakane
		A	B	C						
	isomer)									
2029	perilla alcohol	-	tr	-	-	0.1	0.1	-	tr	0.1
2037	salvial-4(14)-en-1-one	-	-	-	-	0.1	-	-	tr	0.2
2045	humulene epoxide-I	0.1	0.1	0.1	0.1	0.1	0.1	-	tr	0.1
2050	**(*E*)-nerolidol**	**12.5**	tr	**16.3**	0.1	0.2	0.8	-	tr	tr
2051	gleenol	-	-	-	-	-	-	-	tr	tr
2071	humulene epoxide-II	1.2	0.6	1.6	1.0	0.9	0.6	-	0.2	0.7
2079	(*E*)-β-ionone	0.1	-	-	-	-	-	-	-	-
2080	cubenol	-	tr	-	-	0.1	tr	-	tr	0.5
2088	1-*epi*-cubenol	0.1	0.1	0.1	0.1	0.2	0.1	-	0.1	1.0
2096	elemol	-	-	-	-	-	0.4	-	-	-
2098	globulol	0.3	0.2	0.6	-	0.8	-	0.3	-	-
2100	heneicosane	-	-	-	0.1	-	-	-	-	-
2104	viridiflorol	-	-	tr	0.1	-	tr	-	0.1	0.8
2106	guaiyl acetate	-	-	-	-	-	-	-	-	0.2
2123	methyl-4-(4'-methyl-3'-pentenyl)-3-cyclohexenyl ketone[†]	-	-	-	-	-	0.1	-	-	-
2126	3,7-dimethyl-1,7-octadien-3,6-diol	-	-	-	-	-	-	3.4	-	-
2144	rosifoliol	-	-	-	-	0.1	tr	-	-	0.3
2146	spathulenol	0.4	0.6	0.7	0.1	0.9	0.5	1.0	0.1	0.8
2148	(*Z*)-3-hexen-1-yl benzoate	-	-	-	-	0.1	-	-	-	-
2153	neointermedeol	-	tr	-	0.1	-	0.1	-	tr	0.6
2164	6-*epi*-cubenol	tr	tr	0.1	0.1	0.3	0.1	-	tr	0.1
2174	cinnamyl acetate	tr	-	-	-	-	-	-	-	-
2184	*cis*-*p*-menth-3-en-1,2-diol	-	0.1	-	-	0.5	-	-	0.1	-
2185	T-cadinol	0.3	0.1	0.3	0.3	0.8	0.1	-	0.2	1.4
2187	γ-eudesmol	-	-	-	0.2	-	0.9	-	-	5.2
2196	eremoligenol	-	-	-	0.1	-	0.1	-	-	1.7
2206	α-guaiol	-	-	-	-	-	-	-	-	0.5
2209	T-muurolol	0.1	tr	0.1	tr	0.3	tr	-	0.1	0.2
2211	clovenol	-	tr	-	0.1	0.1	-	-	tr	-
2214	torreyol	0.1	tr	0.1	tr	0.2	0.1	-	tr	0.6

RRI*	Compound name	Gauteng					Mpumalanga	Northern Province		Swaziland
		JHB BG (Feb)			Cullinan	Verena	Nelspruit	Lagalametse	Waterberg	Balakane
		A	B	C						
2247	*trans*-α-bergamotol	-	tr	0.1	-	-	-	-	-	-
2250	α-eudesmol	-	-	-	0.6	-	1.1		-	7.5
2255	α-cadinol	0.3	0.1	0.3	-	0.8	-	--	0.2	tr
2257	β-eudesmol	-	-	-	0.8	-	1.4	-	-	3.1
2272	alismol	-	-	-	-	0.4	0.2	-	0.1	0.7
2273	selin-11-en-4α-ol	-	tr	0.2	0.2	0.3	0.1	-	-	0.2
2316	caryophylla-2(12),6(13)-dien-5β-ol (=*Caryophylladienol I*)	-	-	0.1	-	-	-	-	-	-
2324	caryophylla-2(12),6(13)-dien-5α-ol (=*Caryophylladienol II*)	-	0.1	-	0.4	0.3	-	-	0.1	0.4
2328	(*E*,*E*)-10,11-epoxyfarnesyl acetate	-	-	0.4	-	-	-	-	-	-
	3,7,11-trimethyl-10,11-epoxy-2,6-dodecadien-1-yl acetate † (=*10,11-Epoxyfarnesyl acetate*)	1.0	-	-	-	-	-	-	-	-
2350	carvone hydrate† (=*Aralone*)	0.2	-	-	-	-	-	-	-	-
2352	(2*E*,6*E*)-farnesol	-	0.1	0.3	0.3	0.8		-	0.1	-
2375	eudesma-4(15),7-dien-1β-ol	-	tr	0.1	0.1	0.2	0.1	-	0.1	0.5
2385	10-hydroxy calamenene	-	-	0.3	-	0.4	0.1	-	0.1	-
2389	caryophylla-2(12),6-dien-5α-ol (=*Caryophyllenol I*)	-	0.1	tr	0.4	0.4	-	-	0.1	0.2
2392	caryophylla-2(12),6-dien-5β-ol (=*Caryophyllenol II*)	0.2	0.1	0.2	0.4	0.5	-	0.2	0.1	0.1
2438	kaur-16-ene	-	-	-	-	0.2	-	-	-	-
2441	3,7-dimethyloct-1-en-3,6,7-triol †	-	-	-	-	-	-	0.5	-	-
2501	phytol acetate†	-	-	-	-	-	-	-	-	1.1
2503	agglomerone †	-	-	-	-	-	-	-	0.4	-
2622	phytol	-	0.1	0.4	-	0.6	0.2	-	-	2.7
	TOTAL	**92.3**	**97.8**	**93.6**	**97.6**	**93.3**	**95.3**	**72.0**	**97.2**	**89.1**

* RRI: Relative retention indices calculated against *n*-alkanes
% calculated from TIC data
tr = Trace (< 0.1%), † = Tentative identification from Wiley, MassFinder, Adams libraries.



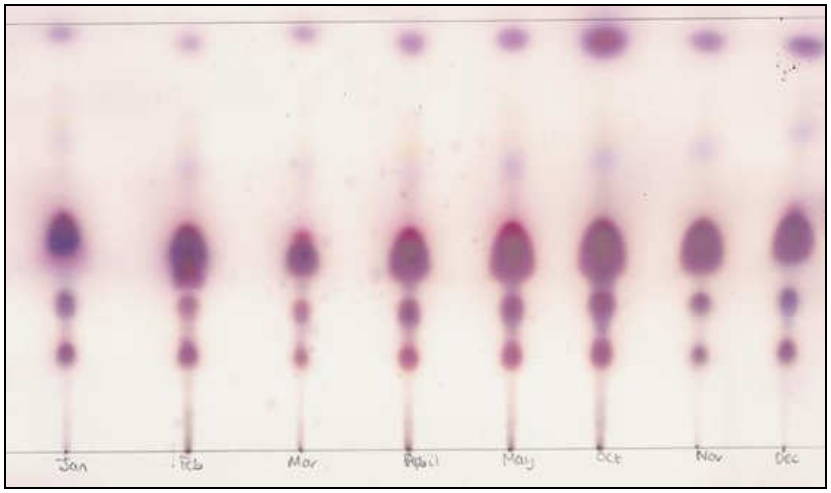
1,8-cineole limonene linalool (*E*)-nerolidol

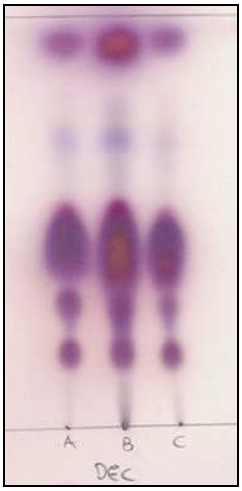
(*Z*)-3-hexenyl nonanoate ß-pinene

Figure 8.4 Chemical structures for major compounds identified in the essential oil of various *H. natalensis* samples.

Seasonal variation: The essential oil yield for the seasonal study on *H. natalensis* varied (0.04-0.2%) with higher yields in the late summer months. Preliminary TLC comparisons were undertaken to investigate if variation in chemical profiles existed between seasonal samples. Similar profiles were obtained for all samples from the three individual trees studied with a representative profile given in Figure 8.5 and Figure 8.6. Confirmation of chromatograms depicting 1,8-cineole and limonene as major constituents were made by comparison with the commercially obtained standards (not shown in Figure 8.5 and Figure 8.6).

The monthly fluctuation of 1,8-cineole and limonene levels in the essential oil showed tree-to-tree variation within a single site as well as variability on a monthly basis (Figure 8.7). The highest tree-to-tree variability for limonene is noted in November for plant B, which contains 4% as opposed to plant A with 26%. Other variations within the individual plant sampling show monthly fluctuations in limonene content, as is the case in May with Plant B having 7% and plant A having 29%. Both these months fall on either side of the winter months and seasonal changes could possibly account for this variation. The total plant to plant variation within a population for limonene was between 4-29% (depicted in blue, Figure 8.7).





A B C
December

Figure 8.5 **Figure 8.6**

The TLC chromatograms indicating similar profiles for plant A, sampled monthly (**Figure 8.5**) and (**Figure 8.6)** representing similar profiles for three individual plants within a population.

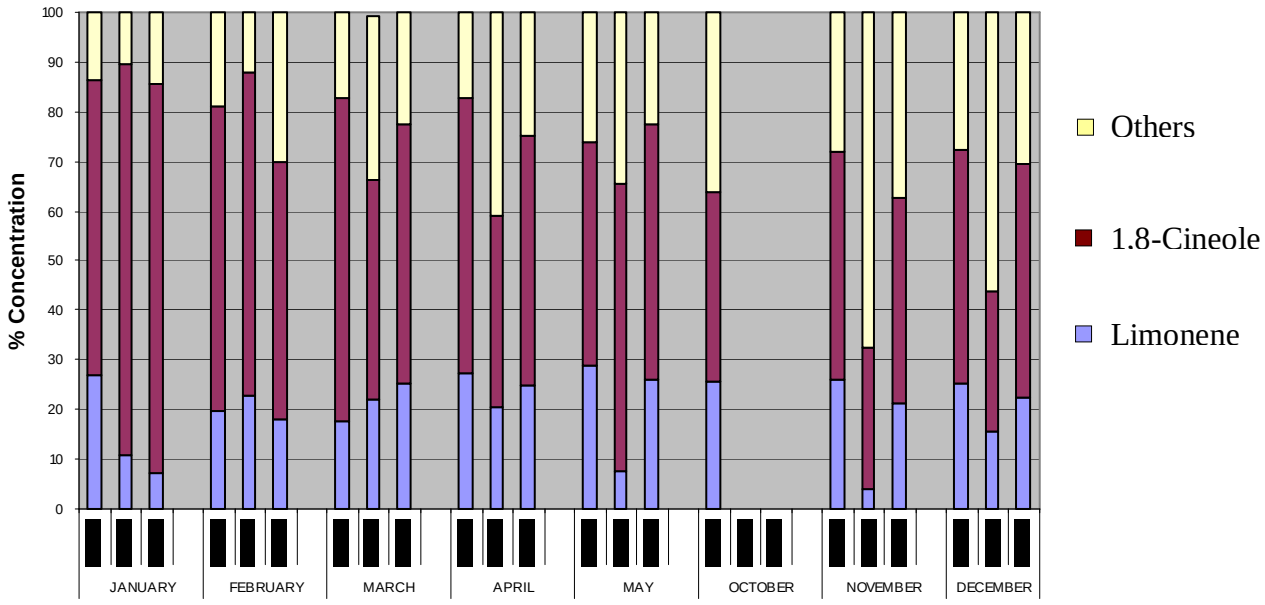


Figure 8.7 The monthly variation of limonene and 1,8-cineole recorded for three individual *H. natalensis* plants at the same locality.

Fluctuation of 1,8-cineole within plant populations showed the highest variation for January (plant A: 60% and plant B: 79%) and December (plant A: 47% and plant B: 28%). Both months fall within the mid summer season. In general plant to plant variation within a population for 1,8-cineole was between 13-20% (depicted in red, Figure 8.7).

The concentration of limonene in plant A has shown an 11% seasonal variation throughout the period of analysis. 1,8-Cineole, indicated higher concentrations in January, February, March, and April (55-65%), with lower concentrations obtained for May, October, November and December, (38-47%). The overall seasonal variation for 1,8-cineole in plant A was 18%.

For plant B (Figure 8.7), the concentration of limonene was low in January (11%), increased in February, March, and April (21-23%), and decreased again in May, November and December (4-15%). 1,8-Cineole had higher concentrations in January and February (65-79%), with concentrations decreasing in March and April (38-44%), increased in May (57%), and again decreased in November and December (28-29%). The seasonal variation for plant B is 19% for limonene and 51% for 1,8-cineole.

For plant C (Figure 8.7), the concentrations of limonene were lower in January (7%) and February (18%) than in the later months (21-26%), with a seasonal variation of 19%. 1,8-Cineole was found in higher concentrations in January (78%), which decreased in the later months (46-52%), with a seasonal fluctuation of 32%.

The two major compounds (limonene and 1,8-cineole) collectively present in the essential oils of *H. natalensis* made up between 32% (plant B, November) to 90% (plant B, January) of the total composition. One would assume that three plants, growing in close proximity within a population in cultivated conditions (Johannesburg Botanical Gardens) where irrigation is regular, would yield a relatively standard composition. This was not the case in this study where fluctuations of the sum of the two major constituents show up to a 58% variation.

Seasonal variation studies on *H. natalensis* by Weyerstahl *et al.* (1992), on the leaves of plants collected in Zimbabwe had a lower oil yield (0.9%) to that found in this study. More interestingly, however, was the difference in composition. The major compounds found in the Zimbabwean oils in

the summer months were (*E*)-β−ocimene (29%) and linalool (26%). In the winter months limonene (21%) and 1,8-cineole (40%) were dominant. The GC-MS analysis of the three February samples (A, B and C) from JHB BG indicates that these compounds were found in negligible quantities. (*E*)-β−Ocimene was absent in plant A, with minor constituent composition 0.3% and 0.1% in plants B and C respectively. Linalool, although present as major constituents in other geographical regions, were only present as a minor constituent here representing 0.1%, 1.2% and 0.5% for plants A, B and C respectively. This study also noted that limonene and 1,8-cineole, both major constituents, were consistently predominant throughout the winter and summer months. In another seasonal study, also undertaken in Zimbabwe (Chagonda *et al.*, 2000), linalool (26.5%) and 1,8-cineole (37.2%) were found to be the major constituents in winter for both the wild and cultivated plants. In the summer months, linalool (26.5%), limonene (13.3%) and 1,8-cineole (24.7%) were seen as major components. For this study, limonene concentrations for the three separate plants within the month of February varied between 16.5-25.4%. 1,8-Cineole composition varied between 23.9-41.2%. These results correlate more closely with the study undertaken by Chagonda *et al.* (2000), than when comparatively examined with the study done by Weyerstahl *et al.* (1992). The average climate for Harare, Zimbabwe (information obtained from an eight year mean, http://www.climate-zone.com/climate/zimbabwe/celsius/harare.htm), indicated very little temperature variation when compared to the seasonal sampling in this study. The overall variation for the Zimbabwean oils was between 2-5 ºC with more variation in the winter months. For the South African oils studied here, a temperature variation of 1-7.5% was noted with the greatest difference found in May, also being a cooler month of the year. Thus, one may deduce that it is unlikely that essential oil variation for *H. natalensis* is determined by climatic conditions.

Geographical variation: The chemical diversity noted between the Zimbabwean *H. natalensis* essential oils and that noted from this study brought about the postulation that geographical distribution might play a role in composition variability. This geographical variability is not novel and can be noted in studies on *A. afra* (Viljoen *et al.*, 2006) and *Eriocephalus* species (Njenga *et al.*, 2005) where the oil composition varied quantitatively and qualitatively within and between natural populations and showed little or no correlation to geographical distribution. A study investigating the geographical variability within South African *H. natalensis* populations was hence undertaken. The geographical oil yield of *H. natalensis* from six different localities showed little variation (0.1-

0.2%) with only the sample from Cullinan having a somewhat higher yield (0.3%). The oil compositions are given in Table 8.4.

The percentage composition of the essential oil from *H. natalensis* representing seven different localities was studied and a dendrogram (Figure 8.8) was generated using the 173 different chemical compounds identified by GC-MS (Table 8.4). Four main types were identified with two groups closely related with a similarity coefficient of 0.8-1. The *H. natalensis* sample from Lagalametse is distinctly different from all other samples, where the characteristic major constituents limonene and 1,8-cineole are absent. (*Z*)-3-Hexenyl nonanoate predominantly present (16.0%) was absent from all other *H. natalensis* samples. Also uniquely different was the *H. natalensis* sample from Balakane having β-pinene (25.2%) as a major constituent. The samples from Gauteng are similarly clustered all having limonene and 1,8-cineole as major constituents. These major compounds were also present in the Nelspruit and Waterberg samples. In addition to these major compounds, linalool was present as a major constituent in the Nelspruit sample, distinguishing it from Gauteng and Waterberg.

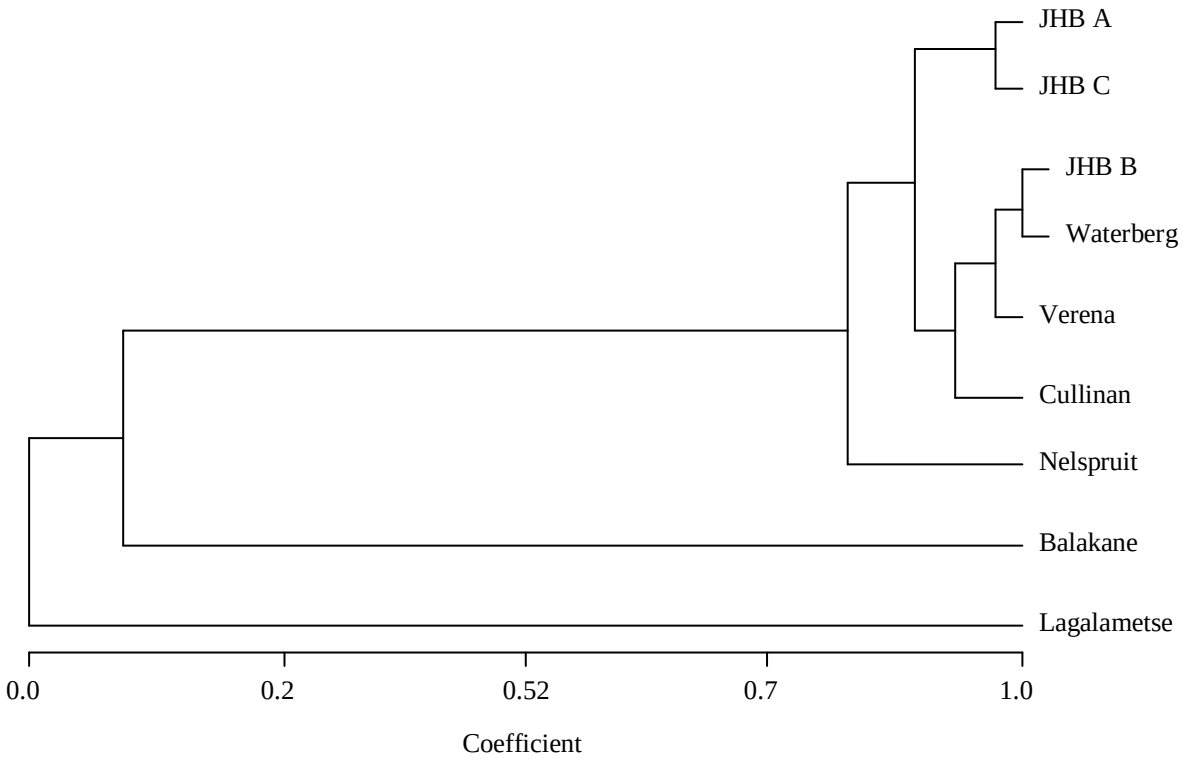


Figure 8.8 Dendrogram constructed from the essential oil data matrix (Table 8.4) for *H. natalensis*.

8.6.2 Antimicrobial activity

Seasonal study: As the chemical variation may impact on the antimicrobial efficacy of the plant, an antimicrobial study (MIC method) was undertaken on the oil samples to determine if antimicrobial variability is present. The acceptable MIC control ranges for all pathogens except *M. catarrhalis* have been previously determined (Chapters 2, 3 and 4) either by the NCCLS (2003), Andrews (2004), or standards determined within the Department of Pharmacy and Pharmacology, University of Witwatersrand. The MIC control ranges for *M. catarrhalis* was set at a range of 0.3-0.6 µg/mL for ciprofloxacin as determined by the mean of ten repetitions. Results for 16 of the 22 oil samples against eight pathogens are represented in Table 8.5. Seasonal samples for January (plant C), February (plant A), March (plant B), May (plant A and B) and November (plant B) were not evaluated by the MIC assay due to insufficient oil sample.

With seasonal variation, one would expect the biological activity to correlate with the fluctuating levels of the major constituents. No trend exists where the two major constituents (1,8-cineole and limonene) show similarities with certain seasonal conditions. This is also seen with the microbiological assessment. Most oil samples show similar antimicrobial patterns over the season with slight variations for some samples. These variations were sporadic and restricted to individual plant samples unrelated to climatic conditions. For the Gram-positive test organisms *S. aureus, B. cereus* and *E. faecalis*, the average MIC values obtained were 6.4, 3.3 and 8.8 mg/mL respectively with the highest variability noted for *S. aureus* with a standard deviation of ± 3.3. Similarly, the Gram-negative test organisms showed little variability with average MIC values of 11.1, 4.8, 8.8 and 5.1 mg/mL for *E. coli, P. aeruginosa, K. pneumoniae* and *M. catarrhalis* respectively. The greatest variability noted amongst the Gram-negative test organisms was recorded for *K. pneumoniae* with a standard deviation of ± 2.9. The yeast, *C. neoformans*, showed the highest sensitivity of all pathogens studied having an average MIC value of 2.1 mg/mL with the least variance (standard deviation of ± 0.3) noted between samples (Table 8.5). The antimicrobial activity for the Zimbabwean *H. natalensis* species (Gundidza *et al.*, 1993) showed good activities against pathogens *E. coli, K. pneumoniae, S. aureus* and *Moraxella* species with lower sensitivities for *P. aeruginosa*. In this study *E. coli* showed poor susceptibility and *K. pneumoniae* moderate activity. Even though the major constituents 1,8-cineole, and limonene, found in the oil studied by Gundidza *et al*. (1993)

correlate with that found in the South African oils, results are not comparable due to the different antimicrobial methods (Gundidza *et al.*, 1993, made use of disc diffusion techniques).

Geographical study: Table 8.6 presents the MIC data obtained when investigating the possibility of antimicrobial related geographical variation. The Gauteng samples (Cullinan, Verena and JHB BG) all indicate consistent moderate antibacterial activity (3.0-16.0 mg/mL). Only *C. neoformans* displayed higher sensitivities (0.8-2.0 mg/mL). Nelspruit, Waterberg and Balakane *H. natalensis* samples also displayed similar antimicrobial efficacies. This was clearly demonstrated when testing these samples against *E. faecalis* (Figure 8.9).

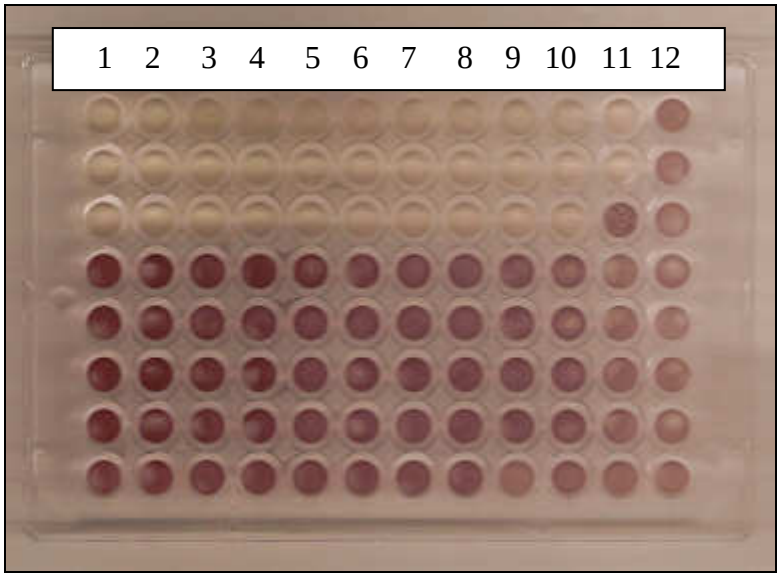


Figure 8.9 The MIC plate for *E. faecalis* showing uniform microbial inhibition for selected *H. natalensis* samples.

Key to Figure 8.9
Columns 1 and 2 = Waterberg[*1]
Columns 3 and 4 = Verena[*1]
Columns 5 and 6 = Nelspruit[*1]
Columns 7 and 8 = JHB BG (sample Feb B)[*1]
Columns 9 and 10 = Swaziland[*1]
Column 11 = Positive control (ciprofloxacin)[*2]
Column 12 = Negative control (having no test substance) showing no inhibition.

[*1] Starting concentration 128 mg/mL.
[*2] Stock antibiotic (ciprofloxacin) solution starting concentration 0.01 mg/mL.

Table 8.5 The mean MIC (mg/mL) for selected *H. natalensis* essential oils for January – December, 2004.

Plant sample	***Staphylococcus aureus* ATCC 12600**	***Bacillus cereus* ATCC 11778**	***Enterococcus faecalis* ATCC 29212**	***Escherichia coli* ATCC 11775**	***Pseudomonas aeruginosa* ATCC 9027**	***Klebsiella pneumoniae* ATCC 13883**	***Moraxella catarrhalis* (clinical strain)**	***Cryptococcus neoformans* ATCC 90112**
January A	12	5	8	11	6	16	8	3
January B	7	4	8	11	4	8	5	2
February B	5	6	8	11	4	8	11	2
February C	5	2	8	11	4	6	4	2
March A	12	3	8	8	4	8	4	2
March C	3	3	8	11	4	8	4	2
April A	4	3	8	11	4	8	4	2
April B	2	3	8	11	4	8	4	2
April C	5	3	8	13	4	8	4	2
May C	4	3	13	11	4	8	4	2
October A	6	3	8	11	4	8	4	2
November A	4	3	16	11	4	8	4	2
November C	11	2	8	11	4	8	4	2
December A	11	5	12	11	2	16	6	3
December B	5	1	6	13	12	6	5	2
December C	7	3	6	11	8	8	7	2
Annual mean	6.4 ± 3.3	3.3 ± 1.2	8.8 ± 2.6	11.1 ± 1.1	4.8 ± 2.3	8.8 ± 2.9	5.1 ± 2.0	2.1 ± 0.3
Control *	0.30×10^{-3}	0.30×10^{-3}	1.25×10^{-3}	0.10×10^{-4}	0.30×10^{-3}	0.60×10^{-3}	0.50×10^{-3}	1.60×10^{-3}

* Ciprofloxacin and amphotericin B served as controls for bacteria and yeasts respectively

The antimicrobial geographical variation was most apparent when observing the *E. faecalis* MIC data obtained from Cullinan (16 mg/mL) and Lagalametse (3 mg/mL) accounting for the large standard deviation of ± 3.8, whereas the geographical variation was least apparent when observing the *C. neoformans* MIC data with a ± 0.5 standard deviation.

Table 8.6 The mean MIC (mg/mL) for *H. natalensis* essential oils selected from different localities.

H. natalensis sample	*Staphylococcus aureus* ATCC 12600	*Enterococcus faecalis* ATCC 29212	*Pseudomonas aeruginosa* ATCC 9027	*Klebsiella pneumoniae* ATCC 13883	*Cryptococcus neoformans* ATCC 90112
Cullinan	8.0	16.0	4.0	8.0	0.8
Verena	4.0	8.0	3.0	4.0	1.0
JHB BG [*1]	5.0	8.0	4.0	7.0	2.0
Nelspruit	6.0	8.0	3.0	6.0	1.0
Waterberg	8.0	8.0	4.5	4.0	1.0
Balakane	4.0	8.0	4.0	8.0	0.5
Lagalametse	1.6	3.3	2.0	3.3	0.5
Geographical mean	5.2 ± 2.3	8.5 ± 3.8	3.5 ± 0.9	5.8 ± 2.0	1.0 ± 0.5
Control [*2]	0.3×10^{-3}	1.25×10^{-3}	0.3×10^{-3}	0.6×10^{-3}	1.6×10^{-3}

[*1] Mean of plant B and C harvested in February (included as a comparison with other samples from Gauteng).
[*2]Ciprofloxacin and amphotericin B served as controls for bacteria and yeast respectively.

The antimicrobial MIC data from the seven different localities (Table 8.6) is presented in a dendrogram (Figure 8.10). With the exception of the Lagalametse sample, all microbiological data was closely clustered approximately around the 0.75-0.85 similarity coefficient. Clustering similarities were evident between both the essential oil data matrix (Figure 8.8) and the microbiological data (Figure 8.10) with the Lagalametse sample existing as a distant outlier in both dendrograms.

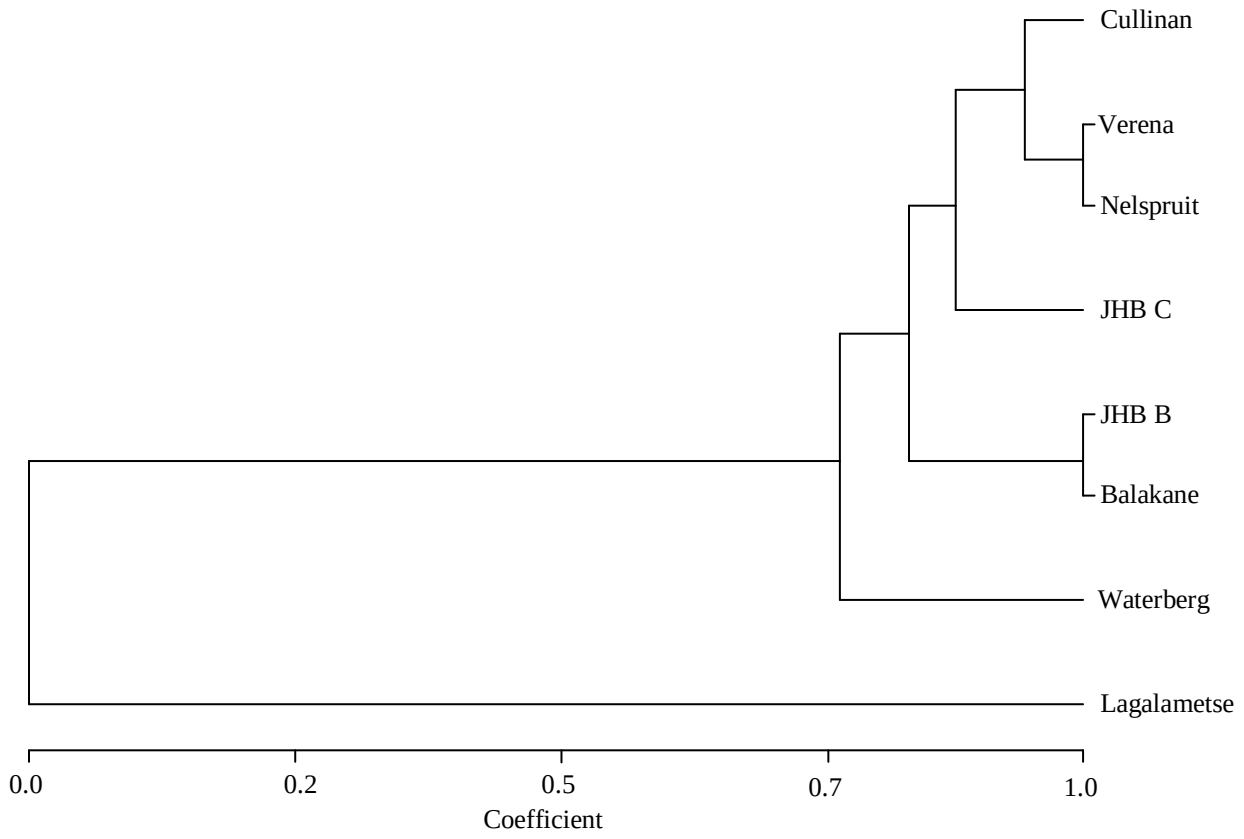


Figure 8.10 Dendrogram constructed from the microbial MIC data in Table 8.6 for *H. natalensis*.

With the exception of the Balakane sample, all geographical samples have 1,8-cineole and limonene as major constituents. ß-Pinene, present as a major constituent only in the Balakane sample, has previously shown either poor (Hinou *et al*., 1989; Kang *et al*., 1992) or moderate (Dorman and Deans, 2000; van Zyl *et al*., 2006) antimicrobial activity against the Gram-negatives. The authors mainly concur that better efficacy was noted for the Gram-positive organisms. With the exception of the MIC results shown for *C. neoformans*, the Balakane sample does not display any distinct antibacterial disparities and therefore it can be deduced that ß-pinene has no major influence on the overall bactericidal activity of the plant, however, the low MIC values (0.5 mg/mL) seen in this sample for *C. neoformans* could possibly be attributed to alterations in mitochondrial and plasma membrane function. Previous studies by Uribe *et al.* (1985) have shown that ß-pinene has an adverse effect on yeast membrane functions. (*E*)-Nerolidol present as a major constituent in both samples A and C from JHB BG have shown poor antimicrobial efficacy in a previous study (van Zyl *et al*., 2006) and thus it can be assumed to have no antimicrobial impact on the *H. natalensis* oils,

however, previous studies on nerolidol have shown that this aliphatic sesquiterpene has a significant role as a penetration enhancer (Xiong *et al*., 1996) and further studies (Brehm-Stecher and Johnson, 2003) demonstrated the non-specific bacterial permeability when nerolidol was combined with polymyxin B. Possibly (*E*)-nerolidol could act synergistically with other *H. natalensis* essential oil components where the compound enhances penetration of other more active molecules. The *H. natalensis* sample from Lagalametse displayed the lowest antimicrobial efficacies of all samples and against all pathogens. The chemistry of the essential oil of the Lagalametse sample is very different from the rest of the samples, having (*Z*)-3-hexenyl nonanoate and linalool as major constituents. A previous study (Dorman and Deans, 2000) has shown that linalool has far greater broad-spectrum antimicrobial activity when comparatively assessed with limonene. The authors presented disc diffusion data where inhibition was greater for linalool against 22 of the 25 test organisms studied. Another study (Carson and Riley, 1995) demonstrated higher efficacies for linalool when comparatively assessed with 1,8-cineole. Higher efficacies were found for linalool in both disc diffusion and MIC methodology. Pattnaik *et al*. (1997) also demonstrated increased efficacies for linalool for 21 of the 30 pathogens studied. A more recent publication (van Zyl *et al*., 2006) has shown increased efficacies against linalool when comparatively assessed with both limonene and 1,8-cineole against four pathogens. This could possibly account for the increased efficacies found in the Lagalametse sample, however, the Nelspruit sample also having linalool as a major constituent (16.7%) indicated lower susceptibilities for the pathogens studied. Thus, (*Z*)-3-hexenyl nonanoate exclusively found in the Lagalametse sample could possibly account for the increased antimicrobial activity noted. (*Z*)-3-Hexenyl nonanoate has been associated with food additives and possibly included into food perishables due to its antimicrobial efficacy, however, this cannot be confirmed and further investigation is recommended on this novel compound.

8.6.3 The enantiomeric influence on microbiological efficacy

It is well-known for several pharmaceutically active compounds that stereochemistry is often related to the specific structure activity relationships of a molecule (Hutt and O' Grady, 1996; van Miert, 2003; Cordato *et al*., 2003). For example, levofloxacin is the optically active L-isomer of ofloxacin. Not only is levofloxin twice as active as ofloxacin but it also has a greater spectrum of antimicrobial activity, being 8-128 times more potent (Morrissey *et al.*, 1996). This resulted in the postulation that when investigating constituent combinations, one should also take into account the enantiomeric

form. With this in mind an investigation was undertaken on the interaction (antagonistic, synergistic, inert or additive) between the two major constituents of *H. natalensis*, with regard to the specific stereochemistry of the optically active molecule i.e. (+), (-) and racemic mixtures of limonene. As indicated in the GC-MS analysis (Table 8.4), TLC profiles (Figure 8.5 and 8.6) and GC composition (Figure 8.7), the major compounds (limonene and 1,8-cineole) identified in the *H. natalensis* oils, predominated both the seasonal and geographical samples, and thus served as a model to study their microbiological interaction further.

Previous studies have shown pathogen selective antimicrobial activity for limonene (Lis-Balchin, *et. al.*, 1996; Neirotti *et al.*, 1996; Dorman and Deans, 2000; Chalchat *et al.,* 2000). For this study the MIC results determined for (+) and (-)-limonene as well as the racemic mixture show that depending on the chirality different microbiological results may be obtained. Analysis of the results outlined in Table 8.7 show that in general the (-)-limonene is the more active component in comparison to the (+)-limonene with higher sensitivities for five of the eight pathogens studied. A disc diffusion study by Dorman and Deans (2000), on the antimicrobial efficacy of different plant volatile oil components indicate moderate to no inhibition for (+)-limonene, depending on the pathogen studied, with highest sensitivity towards *E. coli* (11.2 mm diameter zone of inhibition). Even though the disc diffusion results are not comparable, the general antimicrobial trend for (+)-limonene indicating moderate efficacy was also noted in this study, however, the highest sensitivities found in this study were noted for *B. cereus* and *C. neoformans*, both having MIC values of 3 mg/mL. These pathogens were not investigated by Dorman and Deans (2000). In another study by Chalchat *et al.* (2000), antimicrobial efficacy was greater for the (-)-enantiomer. Conversely to that which was noted here, and with Dorman and Deans (2000), other studies (Lis-Balchin *et al.,* 1996; Aggarwal *et al*., 2002) have indicated higher antimicrobial efficacies for (+)-limonene in the disc diffusion assay. In addition to methodology variation between the literature review and the values reported in this study, further comparative analysis could not be made as Lis-Balchin *et al.* (1996), did not specify which pathogens were investigated. Aggarwal *et al*. (2002), indicated sensitivities only for (+)-limonene, with no values given for (-)-limonene. One common finding in all of the above mentioned studies is that antimicrobial variability between isomers is apparent. The greatest variation in this study was noted for *S. aureus* where there was at least a three-fold difference between the enantiomeric forms of limonene (Table 8.7).

Table 8.7 The mean MIC (mg/mL) for the major constituents limonene and 1,8-cineole independently, and in combination with FIC (in brackets), determined for 1:1 combinations.

Constituent	***Staphylococcus aureus* ATCC 12600**	***Bacillus cereus* ATCC 11778**	***Enterococcus faecalis* ATCC 29212**	***Escherichia coli* ATCC 11775**	***Pseudomonas aeruginosa* ATCC 9027**	***Klebsiella pneumoniae* ATCC 13883**	***Moraxella catarrhalis* (clinical strain)**	***Cryptococcus neoformans* ATCC 90112**
(+)-Limonene	13	3	27	11	4	12	8	3
(-)-Limonene	4	3	27	8	4	6	4	2
1,8-Cineole	8	2	23	8	4	8	16	2
1:1 (+) and (-)-Limonene	8 (1.1)	4 (2.6)	8 (0.6)	8 (1.8)	4 (1.0)	8 (2.0)	1 (0.4)	4 (2.3)
1:1 (+)-Limonene and 1,8-Cineole	16 (3.2)	8 (6.6)	16 (1.3)	32 (6.9)	8 (4.0)	16 (3.3)	16 (3.0)	3 (2.5)
1:1 (-)-Limonene and 1,8-Cineole	8 (3.0)	4 (3.3)	8 (0.6)	27 (6.8)	8 (4.0)	16 (4.6)	16 (5.0)	2 (2.0)
1:1 (+/-)-Limonene and 1,8-Cineole	8 (2.0)	4 (1.5)	8 (1.4)	16 (2.5)	8 (4.0)	16 (4.0)	16 (16)	2 (1.5)
Control *	0.30×10^{-3}	0.30×10^{-3}	1.25×10^{-3}	0.40×10^{-4}	0.30×10^{-3}	0.60×10^{-3}	0.30×10^{-3}	1.60×10^{-3}

* Ciprofloxacin and amphotericin B served as controls for bacteria and yeast respectively.

When the racemic mixture was examined, there was no consistent sensitivity pattern. The best antimicrobial activity noted for the racemate in comparison to the two isomeric forms was noted with studies on *M. catarrhalis* where the racemic form had an MIC value of 1 mg/mL and synergistic FIC value of 0.4. The (+) and (-)-limonene had MIC values of 8 mg/mL and 4 mg/mL respectively. This pattern was also noted for *E. faecalis* with MIC values for the racemate at 8 mg/mL and additive FIC value of 0.6. Both the (+) and (-)-limonene exhibited MIC values of 27 mg/mL.

Comparative evaluation of the compounds studied indicated that efficacy was pathogen specific and none of the compounds showed broad-spectrum efficacy. 1,8-Cineole, known for its biological activity (Magiatis *et al*., 2002), demonstrated the highest efficacy of 2 mg/mL against *B. cereus* and *C. neoformans*. The 1:1 combinations of 1,8-cineole with (+), (-) and (+/-)-limonene mostly show an indifferent interaction indicating FIC's between 1 and <4 for 13 samples.

Plant essential oils however, are complex in nature having a number of constituents and antimicrobial efficacy may depend not only on the chirality of the constituent, but also on the distribution ratio of constituents. The biological activities for a number of individual oil constituents have been studied (Hinou *et al*., 1989; Chalchat and Garry, 1997; Griffin *et al*., 1999). Studies have also been conducted on essential oil combinations (Geda 1995; Lachowicz *et al*., 1998). Nakatsu *et al.* (2000) demonstrated a few instances where essential oil constituents show synergistic and antagonistic profiles. A study (Viljoen *et al.*, 2003, Chapter 3) showed that the combination of camphor and 1,8-cineole contributed synergistically to the overall antimicrobial activity of *O. asteriscoides.* A study on the combination of the major constituents in *A. afra* (Chapter 4, Viljoen *et al.*, 2006) has shown that the major constituents and combination thereof were not responsible for the overall activity. Even though some pharmacognostic research has been undertaken on constituent combinations, very little biological activity has been performed on the enantiomeric forms. With this study on *H. natalensis*, results (Figure 8.11 - 8.13) indicate how the chirality may impact on the microbiological activities obtained.

When *S. aureus* was exposed to the various isomeric forms of limonene together with 1,8-cineole, some antagonistic activity was observed for each isomer (Figure 8.11). Where (+)-limonene and 1,8-cineole were combined, the antimicrobial activity was dependent on the

concentration ratios assessed. Antagonistic profiles were observed for all combinations where (+)-limonene was higher. Where 1,8-cineole and (+)-limonene were present in a 1:1 ratio combination a synergistic pattern was observed, with less significant synergistic profiles for most of the ratios where 1,8-cineole is in a higher concentration than (+)-limonene. The (-)-limonene and 1,8-cineole combination show antagonistic profiles for all ratios. Four antagonistic ratios are not plotted on the isobologram as their values are greater than the range expressed. When a racemic mixture of limonene was combined with 1,8-cineole, ratios were predominantly antagonistic with only one ratio (40% limonene and 60% 1,8-cineole) showing an additive or close to additive profile. The mixture of 1,8-cineole with (+)-limonene at a 1:1 concentration shows the highest synergistic efficacy of all the profiles studied against *S. aureus*. Interestingly with the single compound study (Table 8.7), (-)-limonene showed the highest efficacies of the different enantiomers, yet it is when (+)-limonene is combined with 1,8-cineole in the isobologram study that better efficacies, i.e. synergy are noted.

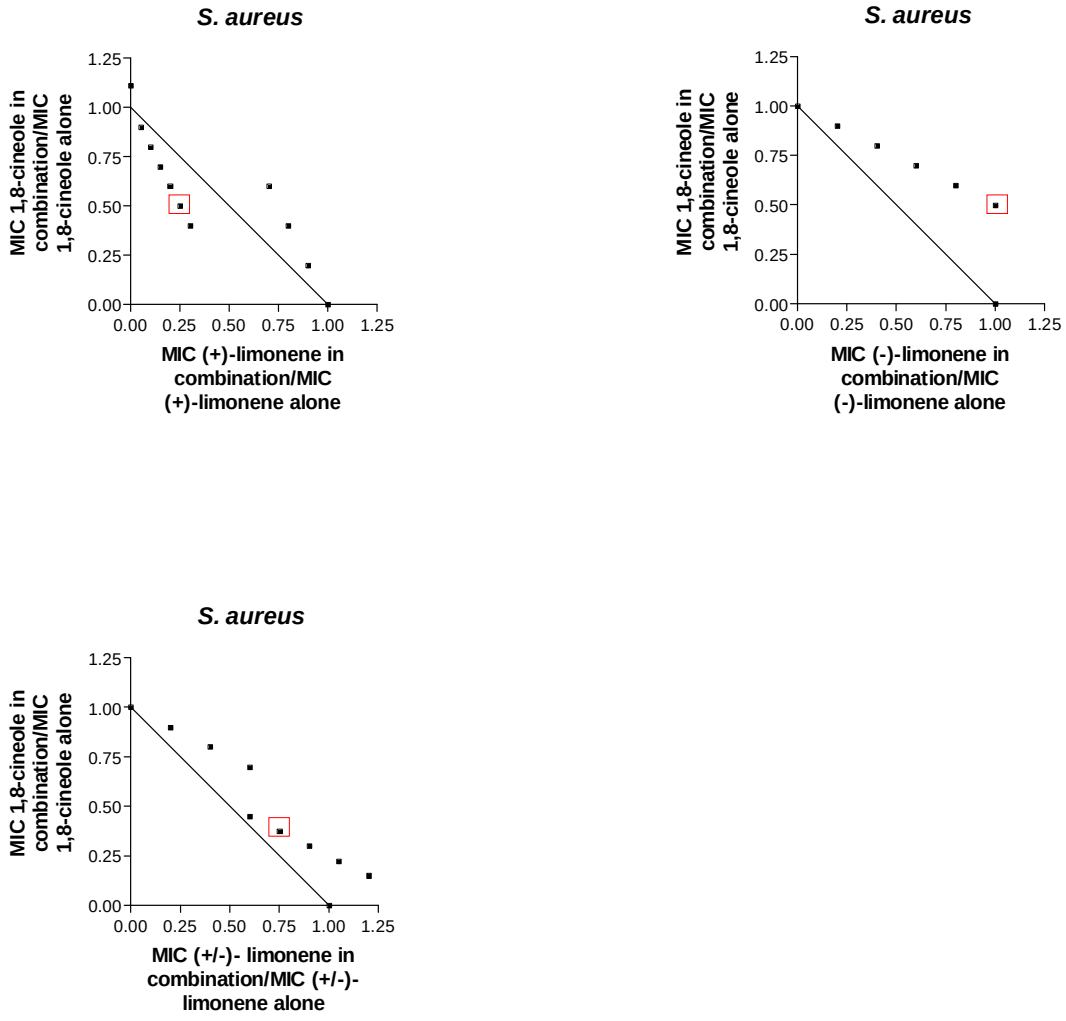
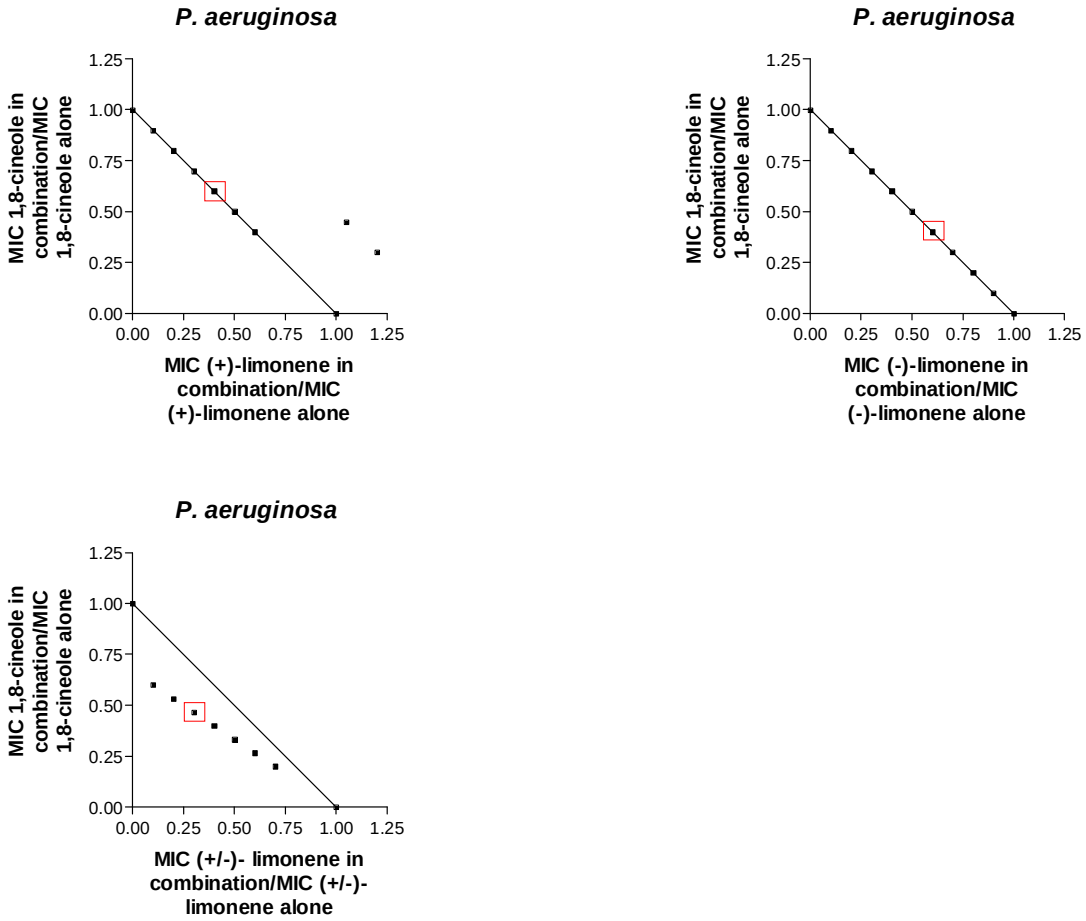


Figure 8.11 Isobologram plots for *S. aureus* (ATCC 12600) when exposed to the combination of (+)-limonene with 1,8-cineole, (-)-limonene with 1,8-cineole and a racemic mixture of limonene with 1,8-cineole. The 1:1 ratio as determined from the raw data is presented as a square.

The isobologram for the Gram-negative pathogen *P. aeruginosa* (Figure 8.12) show mostly additive profiles when both (+) and (-) isomeric forms of limonene were combined with 1,8-cineole. Antagonistic activity is only noted for three ratios where concentrations of (+)-limonene are greater than that of 1,8-cineole i.e. in concentration ratios observed for: 90% (+)-limonene and 10% 1,8-cineole (not noted on isobologram); 80% (+)-limonene and 20% 1,8-cineole; 70% (+)-limonene and 30% 1,8-cineole. Synergy was observed for seven ratios where the racemate of limonene was combined with 1,8-cineole. For the 1:1 MIC study (Table 8.7), an indifferent profile was obtained for all combinations of limonene with 1,8-cineole.



8.12 Isobologram plots for *P. aeruginosa* (ATCC 9027) when exposed to the combination of (+)-limonene with 1,8-cineole, (-)-limonene with 1,8-cineole and a racemic mixture of limonene with 1,8-cineole.

The isobolograms (Figure 8.13) where (+)-limonene and the racemate were combined with 1,8-cineole, and tested against *C. neoformans*, displayed a predominantly additive profile. For the (-)-limonene / 1,8-cineole combination an antagonistic profile exists for most ratios where 1,8-cineole is in higher concentration. With ratios where (-)-limonene predominates (90:10 and 80:10) additive profiles are noted. When observing the MIC data (Table 8.7) of the compounds studied against *C. neoformans*, all 1:1 combinations of limonene with 1,8-cineole show indifference with FIC's 1.5-2.5. These results correlate with the isobolograms where the 1:1 ratio (depicted within the square) is either on or in the close proximity to the straight line.

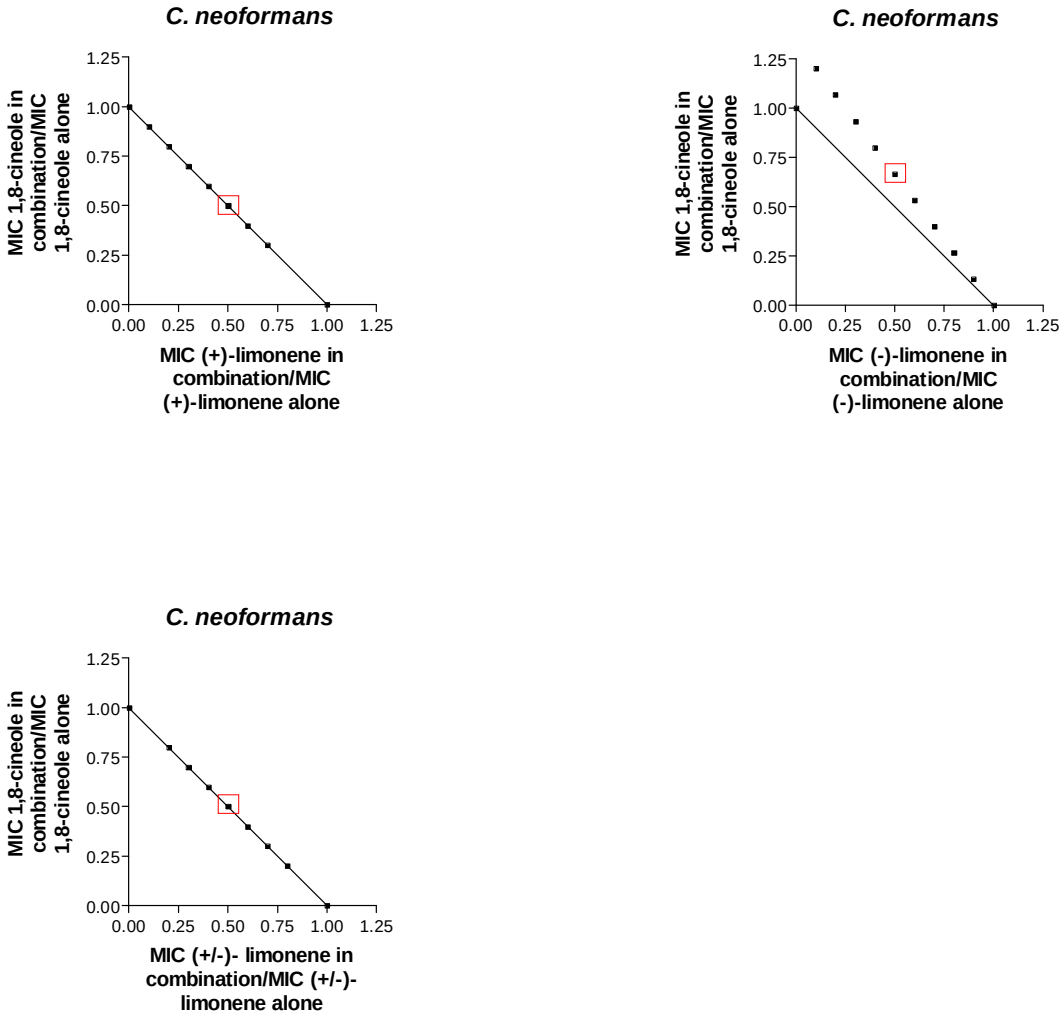


Figure 8.13 Isobologram plots for *C. neoformans* (ATCC 90112) when exposed to the combination of (+)-limonene with 1,8-cineole, (-)-limonene with 1,8-cineole and a racemic mixture of limonene with 1,8-cineole.

From these results obtained with test organisms selected from each microbial group, it is clearly shown that not only may the chirality influence antimicrobial efficacy but also the concentration of the composition may impact on the overall activity. While the independent antimicrobial efficacies of the various enantiomeric forms of limonene highlight the significance of stereochemical configurations for antimicrobial agents, the combination with 1,8-cineole shows that consideration between constituent combinations are just as important. The variation between the (+), (-) and racemic forms of limonene when combined with 1,8-cineole differ from pathogen to pathogen. This was most apparent when observing the racemic mixture of limonene and 1,8-cineole for *S. aureus* where antagonism was noted. For *P. aeruginosa* the racemic mixture indicated synergy and for *C. neoformans* an additive profile was obtained. Variation within the pathogen profile were also apparent with the highest variation noted against *P. aeruginosa* expressing synergy, antagonism and an additive effect depending on the enantiomeric form studied. Such variation may ultimately impact on the overall activity of the whole plant, particularly when trying to correlate structure-activity relationships and must be considered as researchers could be overlooking relevant influences on antimicrobial activities.

This study of *H. natalensis* has shown both the seasonal (with minimal microbiological variation) and geographical characterization as well as enantiomeric impact on biological activity.

8.7 General conclusions

- The major constituents for seven of the nine *Heteropyxis natalensis* samples analysed by GC-MS confirm 1,8-cineole (21.7-41.2%) and limonene (15.0-25.4%) as major constituents. Other major constituents were linalool at 16.7% and 11.4% for Nelspruit and Lagalametse samples respectively. (*Z*)-3-Hexenyl nonanoate (16.0%) was only present in the Lagalametse sample and ß-Pinene (25.2%) was identified as the major constituent in the Balakane sample. (*E*)-Nerolidol was present in the samples JHB BG, sample A (12.5%) and C (16.3%).

- Moderate antimicrobial activity (3.0-16.0 mg/mL) was found for most pathogens with higher sensitivities for *Cryptococcus neoformans*, a respiratory pathogen associated with lung infections. As this plant is traditionally used to treat respiratory disorders, efficacies tested with this pathogen may give credibility to the ethnobotanical use of *Heteropyxis natalensis*.

- The seasonal variation of *Heteropyxis natalensis* samples selected from three trees in the Johannesburg Botanical Garden indicate similar chemical profiles with fluctuation in the quantity of major constituents (1,8-cineole and limonene). The antimicrobial study indicated little variation between samples.

- The geographical variation of *Heteropyxis natalensis* indicates similar major constituent profiles for the Gauteng, Nelspruit and Waterberg samples. The Lagalametse sample showed distinct variation both chemically and microbiologically, and antimicrobial efficacy was higher than in all the other samples.

- The microbiological enantiomeric investigation of the major constituents (limonene and 1,8-cineole) indicate varying activity profiles with (+/-)-limonene in combination with 1,8-cineole having the most significant synergistic ratios against *Pseudomonas aeruginosa. Staphylococcus aureus* also displayed synergistic profiles when investigated with the (+)-limonene / 1,8-cineole combination.

Chapter 9

Tarchonanthus camphoratus L. and *Plectranthus grandidentatus* Gürke, antimicrobial activity and pharmacological interaction of the non-volatile and volatile fractions.

9.1 Introduction

Medicinal plants are often administered through inhalation (Caceres *et al*., 1991; Inouye *et al*., 2001a). This suggests the volatile constituents may (in part) be responsible for antimicrobial activity. Several papers (Brantner *et al*., 1994; Chagonda *et al*., 2000) have focused on the antimicrobial efficacies of either the essential oils or extracts independently. Some papers report on the bioactivity of both volatile and non-volatile plant constituents (Hammer *et al*., 1999; El-Shazly *et al*., 2004; van Vuuren *et al*., 2006), however, when dealing with aromatic plants, there is a need to determine the specific contribution of both the volatile and non-volatile constituents to test the hypothesis that the aromatic constituents, while independently may exhibit poor activity, in combination with non-volatile constituents act synergistically to enhance biological activity.

This study aims to verify the need for the co-existence of volatile and non-volatile constituents for enhanced antimicrobial efficacy using two plants *Tarchonanthus camphoratus* L. (Asteraceae and *Plectranthus grandidentatus* Gürke (Lamiaceae) as a model for further investigation.

Tarchonanthus camphoratus, or the wild camphor bush / tree as it is commonly known, is a strongly scented tree (van Wyk *et al*., 1997; Watt and Breyer-Brandwijk, 1962). The name "anthos" from *Tarchon*- "*anthus*" is derived from the Greek word meaning flower, whereas the name "*camphoratus*" refers to the strong camphor odour, which the leaves impart (van Wyk *et al*., 1997). Hutchings and van Staden (1994), mentions *T. camphoratus* for its potential antimicrobial properties. A literature search on *T. camphoratus* yielded only four articles. One published more or less at the same time as Hutchings and van Staden's article, reporting the volatile constituents of *T. camphoratus* in Kenya (Mwangi *et al*., 1994). Another later publication (McGaw *et al*., 2000) evaluated the biological properties of *T. camphoratus* extracts

together with a number of other species and found no antimicrobial activity when tested at a starting concentration of 12.5 mg/mL. A recent publication (Matasyoh *et al.*, 2007) indicated marked activity in the disc diffusion assay and conflicting poor activity in the MIC assay for selected pathogens tested against the essential oil of *T. camphoratus*. Some taxonomic revision has also been undertaken on the *camphoratus* complex (Herman, 2002).

The large *Plectranthus* genus (over 350 species), of which 300 species occur in Africa, have a rich diversity of ethnobotanical use. A number of reports on the antimicrobial activity have been documented. In a study by Rabe and van Staden (1998), the crude leaf extracts of a number of *Plectranthus* species were investigated for their potential antimicrobial activity. Some of the species i.e. *P. ambiguus, P. ciliatus, P. ecklonii, P. ernsti, P. fruiticosus, P. hadiensis, P. hilliardiae, P. laxiflorus, P. madagascariensis, P. oertendahli, P. oribiensis, P. petiolaris, P. purpuratus, P. reflexus, P. rubropunctatus, P. strigosus, P. succatus, P. verticllatus* and *P. zuluensis* as investigated by disc diffusion assay, exhibited variable antimicrobial activity. A further study undertaken by Maistry (2003), investigated the essential oil composition and antimicrobial activity of eight species (*P. ciliatus, P. fruticosus, P. grandidentatus, P. hadiensis, P. neochilus, P. porphyranthus, P. venteri* and *P. zuluensis*). While Rabe and van Staden (1998) focused on the *Plectranthus* extracts and Maistry (2003) examined the essential oil fraction, it is clear that both studies indicated activity for the Gram-positive test organisms albeit in the disc diffusion assay. From the numerous literature reports on the *Plectranthus* species, it was noted that *P. grandidentatus* has been poorly investigated. In a review article on the *Plectranthus* species (Abdel-Mogib *et al.*, 2002), seven species were noted for their medicinal properties. *Plectranthus grandidentatus* however, was not mentioned. In a more recent review (Lukhoba *et al.*, 2006), the species *P. grandidentatus* was listed within the medicinal ethnomedicinal category. Maistry (2003) did indicate some antimicrobial activity for this species but this was not further explored. Even though antimicrobial assessment was undertaken for *P. grandidentatus* by Teixeira *et al.* (1997), the antimicrobial quantitative values were only reported for the isolated compounds.

This Chapter focuses not only on the antimicrobial properties of the essential oil and extracts of *T. camphoratus* and *P. grandidentatus*, but also on whether pharmacological interactions between the volatile and non-volatile fractions are evident.

9.2 Botanical description

Tarchonanthus camphoratus is a small shrub-like tree having thin oblong leaves which are dark green on the upper side and pale velvety-gray on the underside (van Wyk *et al.*, 1997). The tree is semi-deciduous growing approximately two to nine metres in height. Small whitish flowers are produced giving rise to woolly fruits (van Wyk *et al.*, 1997), as clearly noted in Figure 9.1. These woolly fruits, produced mostly from March through to June are strongly scented (van Wyk and Gericke, 2000).

Plectranthus grandidentatus is classified as a perennial semi-succulent herb. The leaves of *P. grandidentatus* show size variation with ovate to broadly ovate characteristics. The stems have a trailing, dishevelled appearance growing up to two metres (Codd, 1985). White to purple flowers are borne on terminal lateral shoots (Figure 9.3).



Figure 9.1 The leaves of *Tarchonanthus camphoratus* with woolly fruits.

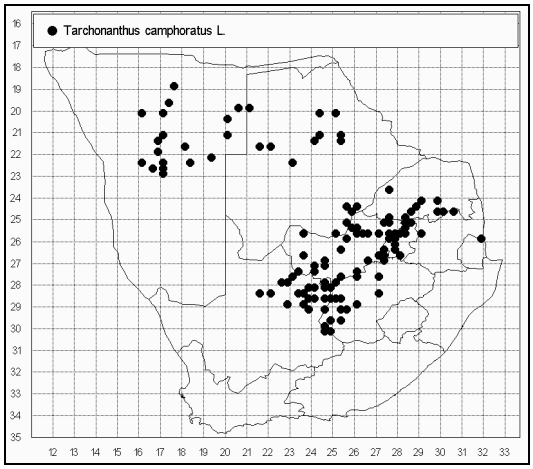


Figure 9.2 The geographical distribution of *T. camphoratus* in southern Africa (SANBI).

9.3 Distribution

Tarchonanthus camphoratus is widely distributed in southern Africa (Figure 9.2), extending from Namibia to Botswana, from the Northern Province, Gauteng, Free State to the Northern Cape (van Wyk *et al.*, 1997; Herman, 2002). The *T. camphoratus* complex has recently been revised, where a number of different taxa have been reclassified due to differences in

morphology and geographical distribution (Herman, 2002). Based on locality and voucher material, *T. camphoratus* L. was the taxon identified for this study.

Plectranthus grandidentatus is naturally occurring within dry, rocky areas or in open woodland. The species is distributed along the eastern region of South Africa (Figure 9.4) from the Soutspansberg mountain range, northwards to Mpumalanga and then through to Swaziland, KwaZulu-Natal Midlands and extending through to the eastern area of the Cape (Codd, 1985).



Figure 9.3 The inflorescence of *Plectranthus grandidentatus*.

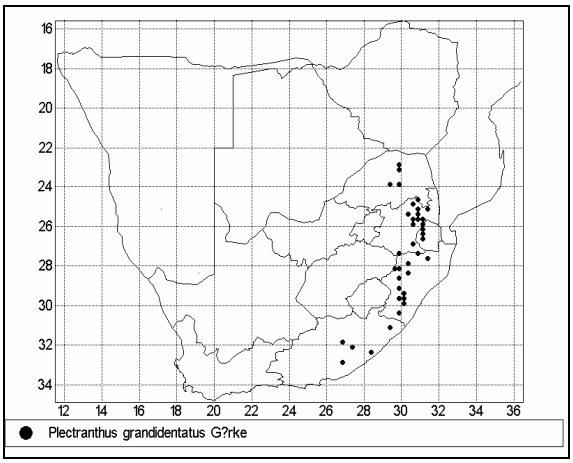


Figure 9.4 The geographical distribution of *P. grandidentatus* in South Africa (SANBI).

9.4 Medicinal uses

The leaves of *T. camphoratus* have been used for numerous medicinal applications, from an infusion for stomach ailments and bronchitis to the inhalation for headaches. Also noted was the use of the leaves as a hot poultice for chest complaints. Chewing of the leaves is said to alleviate toothache (Hutchings *et al*., 1996). The green branches are burnt and the smoke emanating from this is inhaled for sinuses (van Wyk and Gericke, 2000). The use of the plant as a treatment for sexually transmitted diseases has also been noted (Watt and Breyer-Brandwijk, 1962). Africans have used the burned leaf and seed for fumigation and as an anointment in funeral rituals. Interestingly the word “Tarchos” is derived from the Greek word meaning funeral flower.

The ethno-medical information available on the *Plectranthus* species indicates its use for coughs, colds and chest complaints, and administered by means of an infusion made from crushed leaves (Hutchings *et al.*, 1996). Root deconcoctions are prepared for respiratory complaints and abdominal disorders (Watt and Breyer-Brandwijk, 1962). Fifteen species have been identified for the treatment of respiratory conditions and twenty-one species have been categorized for digestive complaints encompassing stomach-ache, diarrhoea and oral infections (Lukhoba *et al.*, 2006). In the same review publication, 20 species were recorded as being used for dermal applications where some species are used as a dressing on wounds, burns and skin ulcerations. The medicinal use of this genus encompasses a number of species, however, very little specific medicinal properties could be found for the species *P. grandidentatus.* In the extensive review of the ethnobotanical literature of the *Plectranthus* species, where over 200 references are cited, only one reference (Cerqueira *et al.*, 2004) refers to the medicinal properties of *P. grandidentatus*.

9.5 Methods

9.5.1 *Chemical aspects*

Plant collection and distillation of essential oils: The aerial parts of *T. camphoratus* and *P. grandidentatus* within a single population were collected from the Walter Sisulu Botanical Garden during February. Voucher specimens (Table 9.1) were deposited within the Department of Pharmacy and Pharmacology, University of Witwatersrand. The plants were hydrodistilled as described in Chapter 2.5.1, however, distillation time was extended by one hour to allow for the complete removal of all volatile constituents from the plant material.

Table 9.1 Plant collection data for *T. camphoratus* and *P. grandidentatus*.

Plant	Voucher	Material distilled (g)	Essential oil yield (% w/w)
T. camphoratus	SVV1100	1243.0	0.10
P. grandidentatus	SVV1099	4.6	0.02

Gas chromatography combined with mass spectrometry (GC-MS) data: Oil samples were quantitatively analyzed with GC-MS using the Hewlett-Packard 1800A GCD system operating under the same conditions as described in Chapter 2.5.1.

Extract preparation with soxhlet apparatus: Plant samples were divided into three quantities for separate extraction procedures with the soxhlet apparatus. The soxhlet extraction method was selected on the basis of the method being heat activated, thus being more comparable to distillation than cold extraction methods. The plant material remaining in the Clevenger apparatus after distillation was dried at 40 °C in an oven (Memmert), weighed (*T. camphoratus* 8.39 g; *P. grandidentatus* 5.96 g) and placed inside an extraction thimble (Whatman). This was placed into a soxhlet extractor (Figure 9.5). The extractor was attached to a flask containing 250 mL of a 1:1 methanol and chloroform mixture. The solvent was selected for its ability to extract both polar and non-polar constituents. Heating of the solvent allowed for the extraction of plant material by movement of the solvent through the outer chamber of the soxhlet apparatus, through to the condenser, after which permeation into the extraction thimble holding the plant material occurred. When the solvent in the soxhlet chamber (in contact with plant material) was the same colour as the pure solvent, extraction was complete (approximately three hours). The operating temperature of the soxhlet extraction process was maintained at approximately 100 °C, thus allowing for both the chloroform (boiling point 61 °C) and methanol (boiling point 65 °C) to extract a full range of constituents. Thereafter, excess solvent was removed by using a rotary evaporator. A second portion of plant material (*T. camphoratus*, 14.80 g; *P. grandidentatus*, 23.30 g) was similarly extracted but with fresh plant material. The third portion of fresh leaf material was oven-dried at 37 ºC for 24 hr. Thereafter, the plant material (*T. camphoratus*, 7.90 g; *P. grandidentatus*, 6.18 g) was placed in a soxhlet apparatus and extracted in the same way as mentioned above. The different preparative techniques of the plant samples allowed for the examination of the non-volatile and volatile constituents. The methods and resultant constituents with a relevant key are as follows:

- EO = Essential oil prepared by distillation (compounds obtained: volatile constituents only).
- NV = Non-volatile constituents prepared by soxhlet extraction of fresh plant material remaining in the still thus having no (or negligible) volatile constituents.
- FC = Volatile and non-volatile constituents from fresh plant material that have been soxhlet extracted (compounds obtained: non-volatile compounds [polar and non-polar], and essential oil constituents).
- DC = Volatile and non-volatile constituents that have been prepared from dried plant material that have been soxhlet extracted (compounds obtained: non-volatile compounds [polar and non-polar], and essential oil constituents).



Figure 9.5 The soxhlet apparatus.

9.5.2 Antimicrobial aspects

Culture, media preparation and MIC assays were undertaken according to the NCCLS (2003) guidelines, Carson *et al*. (1995) and Eloff (1998a). Comparative assays were performed on eight test mico-organisms. Acetone was used as the antimicrobial solvent for *P. grandidentatus* samples, however, due to the insolubility of the *T. camphoratus* plant extracts in acetone, dimethyl sulfoxide (DMSO, Merck) was used as the solvent for reconstitution. As DMSO exhibits some antimicrobial activity, MIC values equivalent to or greater than that found for the DMSO control were omitted from the data (Table 9.6) and considered not susceptible (NS) at the highest concentration tested (64mg/mL). This was inclusive for *P. grandidentatus* samples having little or no susceptibility. Although DMSO is not the preferred choice of solvent for antimicrobial studies, it is accepted for extract miscibility (Okeke *et al*., 2001; van Es *et al*., 2005; Magee *et al*., 2007) and is used extensively in laboratory procedures (Santos *et al*., 2003).

Synergy / antagonistic interaction: Once the independent MIC values were determined, the interaction between the essential oil containing component of the plant (EO) and the non-volatile (NV) component was investigated based on the hypothesis that efficacy may be enhanced by having non-volatile constituents combined with the volatile constituents. The essential oil, at a starting concentration of 64 mg/mL, was combined in selected ratios with the non-volatile extract at a starting concentration of 64 mg/mL. Isobolograms were constructed based on the methodology as described in Chapter 7.5.2. Assays were conducted in duplicate or triplicate where there was a difference in the correlation of results, and the mean plotted on an

isobologram. Conventional antimicrobials (controls) were run in tandem with assays to assess pathogen sensitivity patterns. These are not shown on isobolograms and were included in all replications. Assay controls having solvent only were undertaken with each pathogen where ratios of acetone (used with essential oil and *P. grandidentatus* extract reconstitution) and DMSO (used with *T. camphoratus* extract reconstitution) were assayed to determine the antimicrobial effect of the solvents independently and in various combinations. Raw MIC data for *T. camphoratus* can be noted in Table A3, Appendix A. Raw MIC data for *P. grandidentatus* can be noted in Table A4, Appendix A

Table 9.2 Raw data for the isobologram construction for the volatile (EO) constituent interaction with non-volatile constituents of *T. camphoratus* against *E. faecalis*.

Concentration in mg/mL		MIC values		Ratio values	
X (essential oil)	Y (non-volatile)	X (essential oil)	Y (non-volatile)	X (essential oil)	Y (non-volatile)
16.0	0.0	8.00	0.00	1.000	0.000
14.4	1.6	3.60	0.40	0.450	0.100
12.8	3.2	3.20	0.80	3.200	0.200
11.2	4.8	2.80	1.20	0.350	0.300
9.6	6.4	2.40	1.60	0.300	0.400
8.0*	**8.0***	**2.00***	**2.00***	**0.250***	**0.500***
6.4	9.6	1.60	2.40	0.200	0.600
4.8	11.2	1.20	2.80	0.150	0.700
3.2	12.8	0.80	3.20	0.100	0.800
1.6	14.4	0.40	3.60	0.050	0.900
0.0	16.0	0.00	4.00	0.000	1.000

*The 1:1 ratio as determined from the raw data is presented as a square on the isobolograms.

Table 9.3 Raw data for the isobologram construction for volatile (EO) constituent interaction with non-volatile constituents of *P. grandidentatus* against *S. aureus*.

Concentration in mg/mL		MIC values		Ratio values	
X (essential oil)	Y (non-volatile)	X (essential oil)	Y (non-volatile)	X (essential oil)	Y (non-volatile)
16.0	0.0	2.00	0.00	1.000	0.000
14.4	1.6	0.23	0.03	0.113	0.071
12.8	3.2	0.40	0.10	0.200	0.286
11.2	4.8	0.35	0.15	0.175	0.429
9.6	6.4	0.30	0.20	0.150	0.571
8.0*	**8.0***	**0.25***	**0.25***	**0.125***	**0.714***
6.4	9.6	0.10	0.12	0.050	0.343
4.8	11.2	0.75	0.18	0.375	0.500
3.2	12.8	0.10	0.40	0.050	1.143
1.6	14.4	0.10	0.90	0.050	2.571
0.0	16.0	0.00	0.35	0.000	1.000

*The 1:1 ratio as determined from the raw data is presented as a square on the isobolograms.

For *P. grandidentatus,* the study was limited to three pathogens *S. aureus, K. pneumoniae* and *E. faecalis* due to low oil acquisition. Pathogen selection was based on the ethnobotanical use of *P. grandidentatus*. The raw data for the isobologram construction where the volatile and non-volatile constituents are combined is demonstrated for two test organisms *E. faecalis* (*T. camphoratus*) and *S. aureus* (*P. grandidentatus*) and given in Table 9.2 and Table 9.3 respectively.

9.6 Results and discussion

9.6.1 Essential oil chemistry

Tarchonanthus camphoratus

Ninety-eight compounds were identified in the *T. camphoratus* essential oil, representing 94.3% of the total composition (Table 9.4). Earlier studies on *T. camphoratus* species harvested from Kenya identified 1,8 cineole (16.5%) and α-fenchyl alcohol (29.1%) as major constituents with β-caryophyllene only present in trace amounts (Mwangi *et al*., 1994). In this study α-fenchyl alcohol was not present at all and the bicyclic sesquiterpene β-caryophyllene featured predominantly. A more recent publication by Matasyoh *et al.* (2007), also examined the essential oil from Kenyan *T. camphoratus* species where the major compounds 1,8 cineole (14.3%) and fenchol (15.9%) were identified. β-Caryophyllene was only present as a minor (0.6%) compound. Similarity between both Kenyan species (Mwangi *et al*., 1994; Matasyoh *et al.*, 2007) and that investigated here were the presence of α-pinene (3.9-6.9%) and 1,8-cineole (9.3-16.5%). The two Kenyan studies although harvested from different localities display more similarity. Geographical variation is not uncommon (Viljoen *et al*., 2005 and 2006) and possibly the variance of β-caryophyllene between the Kenyan species and this study can be attributed to differences in locality.

Furthermore, it is interesting to note that although the vernacular name (camphor bush or camphor wood) suggests the presence of camphor, no evidence of such was present in either the analysed oil in this study, nor the *T. camphoratus* oil studied by Matasyoh *et al.* (2007). Camphor was only present in minor quantities (0.38%) in the study undertaken by Mwangi *et al*. (1994).

Table 9.4 Essential oil composition of *T. camphoratus.*

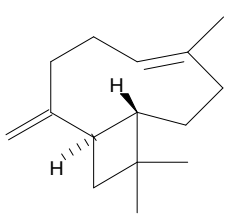
RRI*	Compound name	%
1032	α-pinene	3.9
1035	α-thujene	0.1
1076	camphene	1.7
1118	β-pinene	1.0
1146	δ-2-carene	4.1
1176	α-phellandrene	1.0
1188	α-terpinene	0.1
1203	limonene	1.8
1213	**1,8-cineole**	**9.3**
1246	(*Z*)-β-ocimene	0.1
1255	γ-terpinene	0.1
1266	(*E*)-β-ocimene	tr
1280	*p*-cymene	2.1
1290	terpinolene	0.1
1327	(*Z*)-3-hexenyl acetate	0.3
1360	hexanol	0.4
1391	(*Z*)-3-hexen-1-ol	0.3
1400	nonanal	0.1
1402	(*Z*)-2-hexen-1-ol	0.1
1406	α-fenchone	tr
1450	*trans*-linalool oxide (*Furanoid*)	tr
1466	α-cubebene	0.1
1476	isoledene	tr
1493	α-ylangene	0.4
1497	α-copaene	1.2
1528	α-bourbonene	tr
1535	β-bourbonene	0.5
1544	α-gurjunene	0.1
1553	linalool	0.8
1559	β-maaliene	tr
1571	*trans-p*-menth-2-en-1-ol	tr
1589	β-ylangene	0.8
1601	β-copaene	1.2
1611	terpinen-4-ol	0.7
1612	**β-caryophyllene**	**13.4**
1617	6,9-guaiadiene	0.2
1628	aromadendrene	0.2
1645	cadina-3,5-diene	tr
1659	γ-gurjunene	0.1
1661	alloaromadendrene	3.4
1677	*epi*-zonarene	0.3
1682	δ-terpineol	tr
1687	α-humulene	1.1
1705	γ-muurolene	3.2

RRI*	Compound name	%
1706	α-terpineol	1.0
1708	ledene	0.2
1719	borneol	0.1
1720	4,6-guaiadiene (=γ-*Guaiene*)	0.1
1725	zonarene	1.3
1739	α-muurolene	1.6
1740	valencene	0.7
1744	α-selinene	0.1
1755	bicyclogermacrene	0.2
1773	δ-cadinene	5.1
1776	γ-cadinene	2.0
1799	cadina-1,4-diene (=*Cubenene*)	0.4
1807	α-cadinene	0.6
1853	*cis*-calamenene	1.5
1864	*p*-cymen-8-ol	0.1
1918	β-Calacorene	tr
1941	α-Calacorene	0.5
1943	palustrol	0.2
2001	isocaryophyllene oxide	0.1
2008	caryophyllene oxide	2.2
2017	maaliol	0.1
2029	perilla alcohol	0.1
2033	epiglobulol	0.1
2037	salvial-4(14)-en-1-one	0.2
2057	ledol	0.5
2058	13-tetradecanolide	0.1
2071	humulene epoxide-II	0.1
2072	cubeban-11-ol†	0.4
2074	caryophylla-2(12),6(13)-dien-5-one	0.1
2080	cubenol	1.1
2088	1-*epi*-cubenol	1.8
2098	globulol	1.1
2104	viridiflorol	0.5
2144	rosifoliol	0.6
2146	spathulenol	0.7
2153	neointermedeol	0.2
2164	6-*epi*-cubenol	0.1
2165	1,10-diepicubenol†	0.8
2185	T-cadinol	2.6
2209	T-muurolol	2.0
2214	torreyol	1.0
2247	*trans*-α-bergamotol	0.3
2255	α-cadinol	4.3
2264	intermedeol	0.1
2265	4-(4'-Methyl-3'-pentenyl)-3-cyclohexenyl-propyl ketone	0.1
2272	alismol	0.1
2273	selin-11-en-4α-ol	0.2

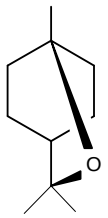
RRI*	Compound name	%
2316	caryophylla-2(12),6(13)-dien-5β-ol (=*Caryophylladienol I*)	0.2
2324	caryophylla-2(12),6(13)-dien-5α-ol (=*Caryophylladienol II*)	0.8
2375	caryophylla-2(12),6-dien-5α-ol (=*Caryophyllenol I*)	0.4
2384	10-hydroxy calamenene	0.3
2389	eudesma-4(15),7-dien-1β-ol	0.3
2392	caryophylla-2(12),6-dien-5β-ol (=*Caryophyllenol II*)	0.1
2622	phytol	0.6
	TOTAL	**94.3**

* RRI: Relative retention indices calculated against *n*-alkanes
% calculated from TIC data
tr = Trace (< 0.1 %)
† = Tentative identification from Wiley, MassFinder, Adams libraries.

Major compounds: The major compounds (Figure 9.6) identified by GC-MS of *T. camphoratus* are β-caryophyllene (13.4%) and to a lesser extent 1,8-cineole (9.3%), representing 22.7% accumulatively.



β-caryophyllene



1,8-cineole

Figure 9.6 Chemical structures for major compounds identified in the essential oil of *T. camphoratus*.

Plectranthus grandidentatus

Sixty-two compounds were identified in the *P. grandidentatus* essential oil, representing 95.6%

of the total composition (Table 9.5). A previous study on the essential oils of numerous *Plectranthus* species displayed quantitative and qualitative variation in major compound composition. Some *Plectranthus* species (*P. ciliatus* ex Ferncliff and *P. zuluensis* ex hort C. Potgieter) did not have T-cadinol and camphor at all. Other species displayed only T-cadinol as both a minor compound (*P. neochilus*, 1.3%) and a major compound (*P. hadiensis*, 26.9%).

Plectranthus hadiensis was the only other species shown to include both T-cadinol and camphor as major constituents (Maistry, 2003).

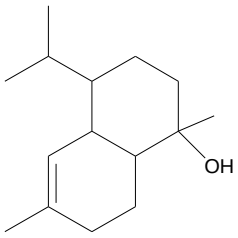
Table 9.5 The essential oil composition of *P. grandidentatus* (Maistry, 2003).

RRI*	Compound name	Percentage
1032	α-pinene	0.6
1035	α-thujene	0.4
1076	camphene	0.2
1132	sabinene	0.8
1176	α-phellandrene	0.7
1188	α-terpinene	0.1
1203	limonene	9.4
1218	β-phellandrene	0.5
1246	(*Z*)-β-ocimene	0.1
1255	γ-terpinene	0.4
1266	(*E*)-β-ocimene	1.9
1280	ρ-cymene	0.6
1290	terpinolene	0.2
1391	(*Z*)-3-hexene-1-ol	tr
1393	3-octanol	0.5
1406	α-fenchone	4.3
1452	1-octen-3-ol	0.6
1495	bicycloelemene	0.3
1505 + 1497	dihydroedulane 2 + α-copanene	0.5
1532	**camphor**	**15.2**
1553	linalool	0.5
1571	*trans*-ρ-menth-2-en-1-ol	tr
1594	*trans*-β-bergamotene	0.1
1600	β-elemene	0.3
1612+1611	β-caryophyllene + terpinen-4-ol	7.7
1628	aromadendrene	0.1
1638	*cis*-ρ-menth-2-en-1-ol	0.1
1655	9-epi-β-caryophyllene	0.6
1661	alloaromadendrene	0.6
1668	(*E*)-β-farnesene	0.1
1687	α-humulene	1.0
1706	α-terpineol	0.6
1719	borneol	1.5
1726	germacrene D	1.2
1737	(*Z*)-*E*-α-farnesene	0.5
1755	bicyclogermacrene	3.3
1764	sesquicineol	9.2
1773	δ-cadinene	0.4
1776	γ-cadinene	1.2

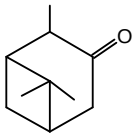
RRI*	Compound name	Percentage
1784	(*E*)-α-bisabolene	0.5
1810	3,7 guaiadiene	0.1
1845	*trans*-carveol	0.1
1853	*cis*-calamenene	0.2
1864	ρ-cymene-8-ol	tr
2001	isocaryophyllene oxide	1.0
2008	caryophyllene oxide	3.9
2050	(*E*)-nerolidol	tr
2051	gleenol	tr
2069	germacrene D-4-ol	0.5
2080	cubenol	5.6
2098	globulol	0.5
2104	viridiflorol	0.3
2144	spathulenol	2.7
2170	β-bisabulol	0.3
2187	**T-cadinol**	**10.3**
2209	T-muurolol	0.2
2232	α-bisabolol	1.9
2247	*trans*-α-bergamotol	0.2
2255	α-cadinol	0.7
2324	caryophyllandienol-2	0.1
2389	caryophyllenol-1	0.2
2392	caryophyllenol-2	0.3
	TOTAL	**95.6**

***** RRI: Relative retention indices calculated against *n*-alkanes
% calculated from TIC data
tr = Trace <0.1%.

Major compounds: The two major compounds (Figure 9.7) in the essential oil of *P. grandidentatus* are T-cadinol (10.3%) and camphor (15.2%), representing 25.5% accumulatively.



T-cadinol



camphor

Figure 9.7 Chemical structures for major compounds identified in the essential oil of *P. grandidentatus*.

9.6.2 Antimicrobial activity

In previous studies with *H. cymosum* subsp. *cymosum* (van Vuuren *et al*., 2006, Chapter 6) and *C. gratissimus* var. *subgratissimus* (Chapter 7), it was observed that the non-volatile constituents in a plant showed higher antimicrobial activity than the volatile constituents. Studies with *H. natalensis* (Chapter 8) have shown that plant essential oil constituents may play a synergistic role. This study on *T. camphoratus* and *P. grandidentatus* examined the interactive role of volatile and non-volatile constituents to determine if when combined, constituents would have antimicrobial synergistic properties. The MIC data for the EO, NV, FC and DC for both *T. camphoratus* and *P. grandidentatus* is presented in Table 9.6.

Tarchonanthus camphoratus

The MIC values for the essential oils (EO) ranged between 1.5-16.0 mg/mL depending on the pathogen studied. These values were significantly higher in activity than that found for the Kenyan *T. camphoratus* oil, exhibiting MIC values within the range 113-450 mg/mL (Matasyoh *et al.*, 2007). Previous studies on β-caryophyllene have shown poor antimicrobial activity when investigated against 16 test organisms (Kang *et al*., 1992). This could possibly indicate that it is not the single major compound β-caryophyllene that is responsible for activity but that a synergistic interaction exists between compounds, or even that one or more of the minor compounds not present in the Kenyan samples could be responsible for the activity noted in the South African species.

The non-volatile fraction (NV) devoid of volatile constituents displayed higher antimicrobial efficacies with a narrow MIC range between 2.0-4.0 mg/mL, with the exception of *E. faecalis* and *K. pneumoniae* showing no susceptibility at the highest concentration (16.0 mg/mL) tested.

Table 9.6 The mean MIC (mg/mL) for the essential oil (EO) and plants that have undergone soxhlet extraction (NV, FC, DC) for *T. camphoratus*[*1] and *P. grandidentatus*[*2].

Pathogen	Essential oil (EO)		Non-volatile (NV)		Combined fresh material (FC)		Combined dried material (DC)		Control[*3]
	T. camp[*1]	***P. grand***[*2]	***T. camp***[*1]	***P. grand***[*2]	***T. camp***[*1]	***P. grand***[*2]	***T. camp***[*1]	***P. grand***[*2]	
Staphylococcus aureus ATCC 25923	6.0	4.0	2.0	0.5	2.0	8.0	1.5	0.16	0.2×10^{-3}
Bacillus cereus ATCC 11778	3.5	3.0	3.0	0.02	4.0	4.0	2.0	0.63	0.2×10^{-3}
Enterococcus faecalis ATCC 29212	NS	12.0	NS	NS	NS	6.0	NS	0.06	0.6×10^{-3}
Escherichia coli ATCC 11775	16.0	NS	3.0	NS	3.5	NS	4.7	NS	0.1×10^{-4}
Pseudomonas aeruginosa ATCC 9027	6.0	4.0	4.0	3.3	4.0	NS	3.3	1.5	0.3×10^{-3}
Klebsiella pneumoniae NCTC 9633	NS	NS	NS	4.0	4.0	8.0	4.0	4.0	0.8×10^{-3}
Candida albicans ATCC 10231	6.0	6.0	2.0	3.0	0.6	8.0	0.3	6.0	2.5×10^{-3}
Cryptococcus neoformans ATCC 90112	1.5	3.0	2.0	3.0	3.0	4.0	1.5	0.13	2.5×10^{-3}

[*3]Ciprofloxacin and amphotericin B served as controls for bacteria and yeasts respectively.
NS = Not susceptible at the highest concentration tested (64mg/mL).

Of the two combined preparations, the dried plant material showed the most favourable synergistic profile with MIC values between 0.3-4.7 mg/mL, depending on the pathogen studied. Comparative investigation of two pathogens (*E. coli* and *K. pneumoniae*) investigated by both McGaw *et al.* (2000) and the combined fractions in this study indicate moderate antimicrobial activity.

Even though each pathogen showed different sensitivity patterns, it is clear that when volatile and non-volatile constituents are combined efficacy is enhanced. This is seen to a lesser extent for the fresh plant material (two pathogens) and more notable for the dried plant material (five pathogens). Although ethnobotanical reports (Hutchings *et al.*, 1996; van Wyk *et al.*, 1997; van Wyk and Gericke, 2000) make more reference to the use of the dried plant material, the traditional use of the plant is not restricted to the dried material and depending on the ethnobotanical use, fresh plant material is also administered.

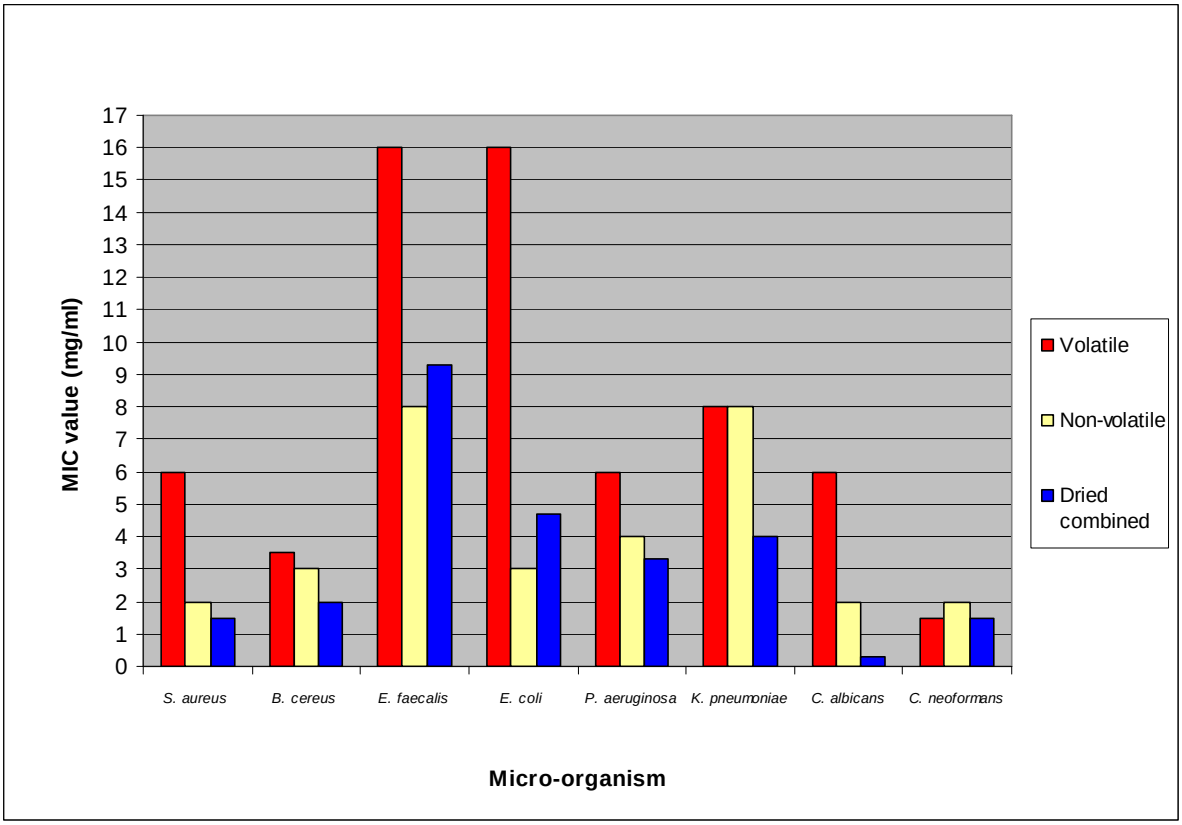


Figure 9.8 The comparative MIC (mg/L) for the volatile constituents (EO), non-volatile constituents (NV) and dried combined (DC) constituents for *T. camphoratus.*

Figure 9.8 shows a condensed version of the MIC results (EO, NV and DC) obtained from Table 9.6, allowing for easier comparative evaluation. The FC plant material and controls have been excluded allowing for the more active combined plant material (DC) to be comparatively assessed with the essential oils and non-volatiles. Results show that for all pathogens with the exception of *C. neoformans* and *K. pneumoniae* where efficacies are equivalent, the non-volatile extracts devoid of any essential oils show higher antimicrobial efficacy than the volatile constituents. For *B. cereus, K. pneumoniae* and *C. neoformans*, both volatile and non-volatile constituents were more or less equal in efficacy. It is interesting to note that two of these organisms (*K. pneumoniae* and *C. neoformans*) are predominantly associated with respiratory infections. *Tarchonanthus camphoratus* is administered by inhalation thus suggesting that the volatile constituents may be the active constituents.

The synergistic roles of the volatile and non-volatile constituents in various ratios are depicted in isobolograms (Figures 9.9-9.11). The control isobolograms for all pathogens showed that when acetone was in higher concentration, no antimicrobial activity was obtained. When DMSO was in greater concentration, antimicrobial activity was minimal. When plotted on an isobologram no comparative ratios were noted thus indicating that the solvents had no marked effect on the results obtained for the test plant material.

The isobolograms for the Gram-positive organisms indicate synergy for most ratios. Both *B. cereus* and *E. faecalis* exhibited antagonism for one ratio where 80% non-volatile constituents are combined with 20% volatile constituents. These ratios are not seen on the isobolograms as the points fall out of the 1.25:1.25 scale (Figure 9.9). This predominantly synergistic pattern suggests that the presence of the essential oils may be necessary for increased efficacy as observed in the ratios where higher essential oil (volatile) content results in synergy. The Gram-positive test organisms *S. aureus* and *B. cereus* show similar synergy patterns in comparison with the MIC study (Table 9.6). No comparison could be made for *E. faecalis* due to lack of susceptibility in the MIC assay.

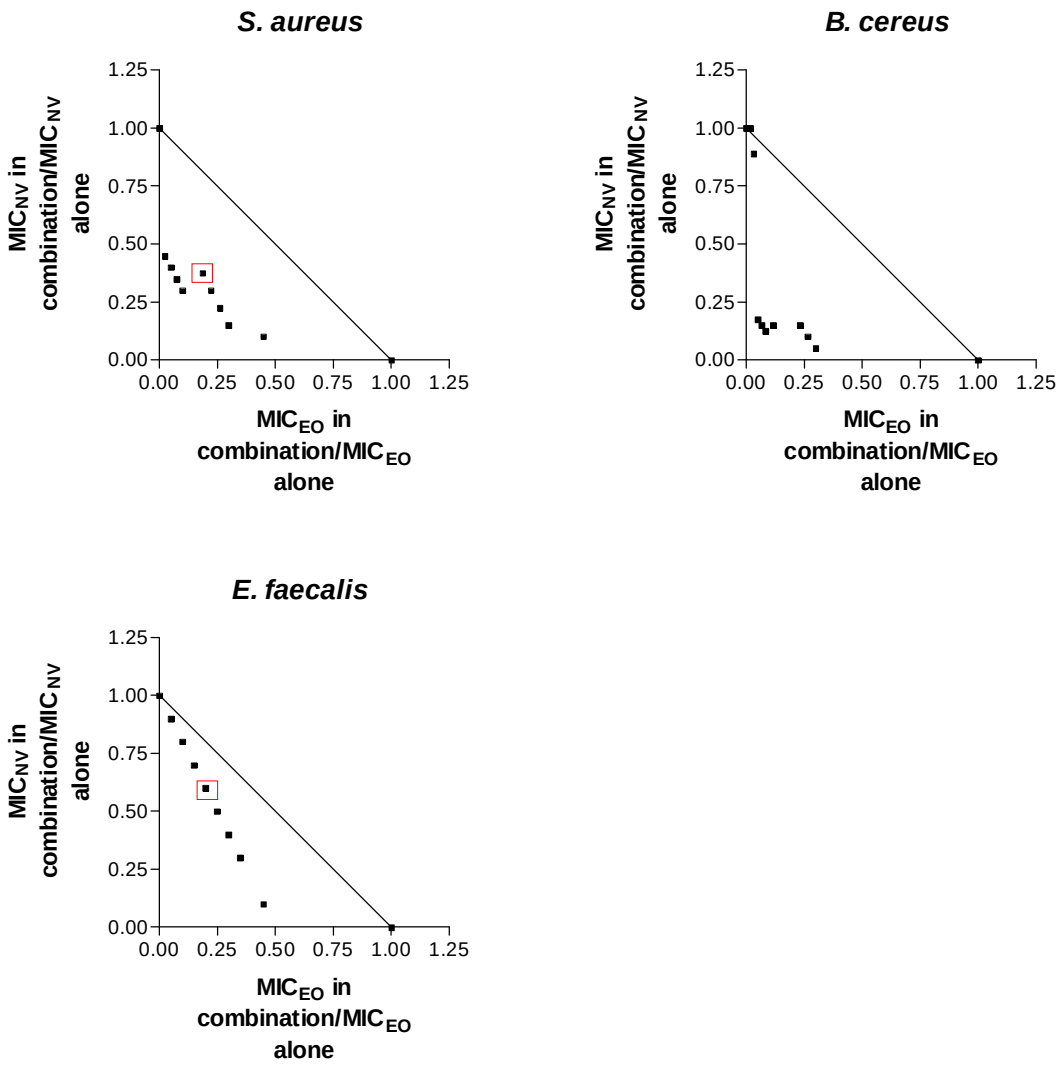


Figure 9.9 The antimicrobial efficacy of the Gram-positive test organisms (*S. aureus, B. cereus* and *E. faecalis* respectively) against varying concentrations of the non-volatile (NV) and volatile (EO) constituents of *T. camphoratus.*

Isobologram studies for the Gram-negative pathogens (Figure 9.10) mostly indicate synergistic interactions. It was only with the isobologram for *E. coli* that antagonism was seen for five ratios (three of which are not seen on isobologram, as the value falls out of the 1.25:1.25 scale). When cross-examining with MIC data, it can be noted that the combined plant material (fresh and dried) showed no increased efficacy with *E. coli* and this was the only pathogen studied which showed this pattern. For isobologram studies with *P. aeruginosa* and *K. pneumoniae*, results were congruent with the MIC values. As noted with *E. faecalis*, similar results were obtained for *P. aeruginosa* where ratios having a higher non-volatile component showed an additive profile

and in ratios having a higher volatile concentration synergy was observed. This supports the need for the presence of volatile constituents in order for synergy to occur. Synergy was observed for all ratios, except one, when studied against the test organism *K. pneumoniae*.

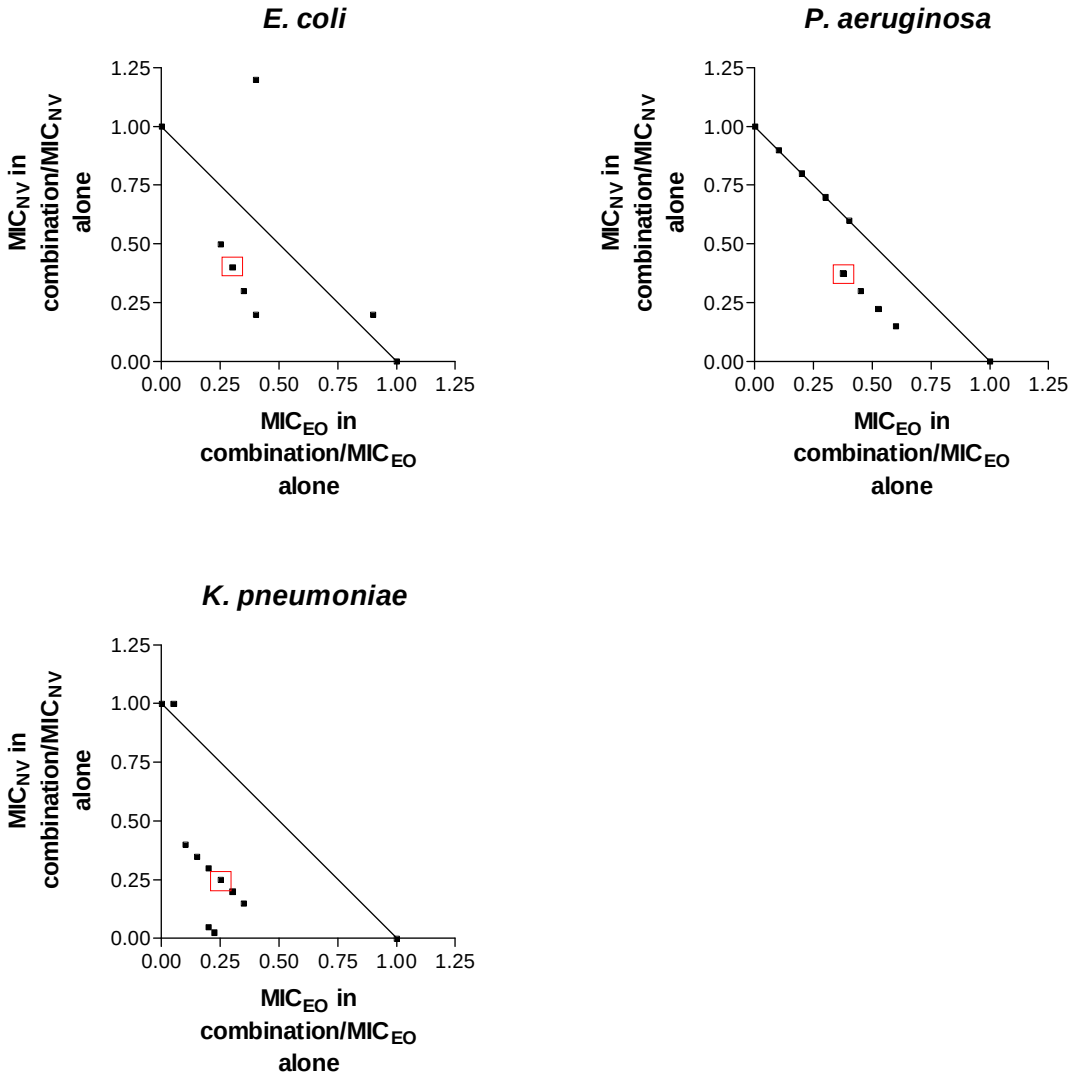


Figure 9.10 The antimicrobial efficacy of the Gram-negative test organisms (*E. coli, P. aeruginosa* and *K. pneumoniae* respectively) against varying concentrations of the non-volatile (NV) and volatile (EO) constituents of *T. camphoratus.*

The isobolograms for the yeasts (Figures 9.11) show synergistic profiles for all ratios studied. This again confirms the need for volatile constituents to enhance activity. Correlation with the

MIC study is true for *C. albicans* where both the fresh and dried combined plant material had higher efficacies than when studied independently.

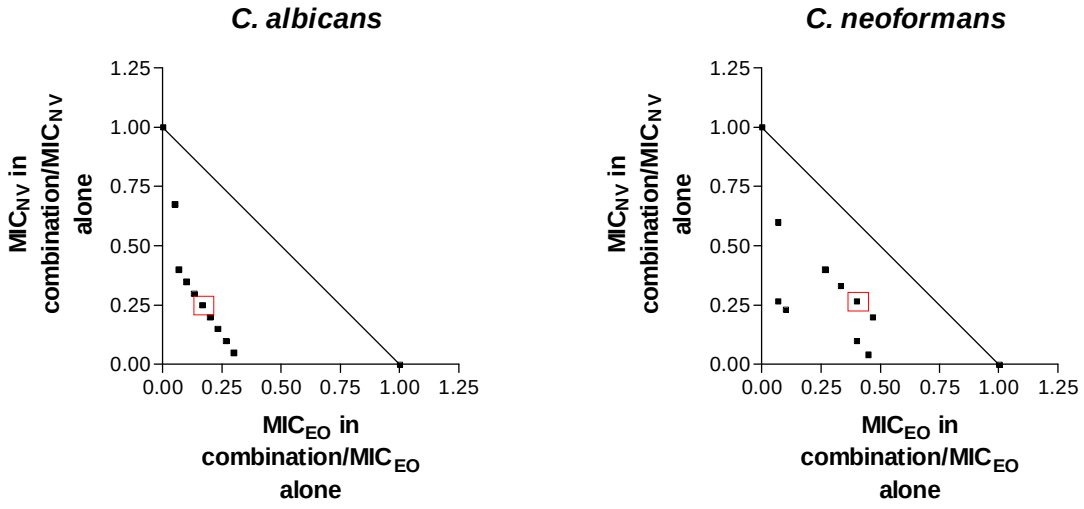


Figure 9.11 The antimicrobial efficacy of the yeasts (*C. albicans* and *C. neoformans* respectively) against varying concentrations of the non-volatile (NV) and volatile (EO) constituents of *T. camphoratus*.

The overall comparative assessment of the volatile, non-volatile with the combined volatile / non-volatile extracts show that for the eight pathogens studied in the MIC assay, five showed increased efficacy for the dried combined material, thus supporting the theory that volatile constituents increase efficacy. This was further validated by the isobologram studies where a predominant synergistic profile for all pathogens was presented. All 1:1 ratios on the isobolograms depicted as a square show synergism for all pathogens including *B. cereus*. Due to the close proximity of the ratio points on the *B. cereus* isobologram, no square was inserted for the 1:1 ratio as this would impair data visualization.

Plectranthus grandidentatus

A number of studies have been undertaken on the chemistry of the *Plectranthus* genus (Batista *et al.*, 1994; Teixeira *et al.*, 1997; Dellar *et al.*, 1996; Gaspar-Marques *et al.*, 2005) and in an excellent review (Abdel-Mogib *et al.*, 2002) where the isolation and identification of antimicrobial compounds amongst others were initiated. From these studies it is clear that the main phytochemical constituents found in the extracts of the *Plectranthus* genus are the

diterpenoids. Approximately 140 diterpenoids have been identified in the leaf extracts of *Plectranthus* species of which vinylogous quinones, acylhydroquinones, dimeric abietanoids and royleanones were isolated from *P. grandidentatus* (Abdel-Mogib *et al.*, 2002). Other compounds isolated include grandidone A and B (Uchida *et al*., 1981) and 7-epigrandidone D (Teixeira *et al.*, 1997). From bio-autographic studies undertaken by Teixeira *et al.* (1997), it was confirmed that the royleanones were the compounds responsible for antimicrobial activity. Previous antimicrobial studies on *Plectranthus* species have shown variable antimicrobial activity (Rabe and van Staden, 1998). Of the four test organisms studied, the greatest antimicrobial activity was found for *S. aureus* against 26 *Plectranthus* species. The other test organisms, *S. epidermidis*, *B. subtilis* and *M. lutea* indicated mostly poor to no activity in the disc diffusion assay. The antimicrobial activity for *P. grandidentatus* was not investigated, possibly due to previous work undertaken on the species (Teixeira *et al.*, 1997). The authors (Rabe and van Staden, 1998) however, did recommend that further antimicrobial investigation be carried out on the essential oil of the species. Table 9.6 presents the MIC data obtained for *P. grandidentatus* essential oils and extracts prepared with and without inclusion of the volatile constituents.

The essential oil MIC data for *P. grandidentatus* (Table 9.6) indicate variable sensitivities, depending on the pathogen studied. Two of the three Gram-positive test organisms indicated moderate to good activity (*S. aureus*, 4 mg/mL; *B. cereus*, 3 mg/mL). Only *E. faecalis* indicated poor sensitivity (12 mg/mL). Of the three Gram-negative organisms investigated, only *P. aeruginosa* showed sensitivity towards the essential oil of *P. grandidentatus* with an MIC value of 4 mg/mL. These results were not congruent with the disc diffusion results as seen with Maistry's (2003) report where antimicrobial efficacy was only noted for *B. cereus* and *C. neoformans*. For the MIC results obtained in this study these two pathogens showed the highest sensitivity patterns. As seen in previous studies and discussed in further detail in Chapter 11.9, disc diffusion methodology is not always comparable to MIC assays. Maistry (2003) did perform a MIC assay with *B. cereus* (4 mg/mL) where results were more or less congruent with those obtained here (3 mg/mL).

Antimicrobial correlation with the essential oil chemistry suggests that possibly the major compound, camphor, may in part be responsible for the antimicrobial efficacy as camphor is known to possess some antimicrobial properties (Griffin *et al*., 1999; Magiatis *et al*., 2002). The bactericidal effect of T-cadinol, also present as a major constituent in the *P. grandidentatus* oil has been previously investigated against *S. aureus* and *E. coli* where results indicated high

susceptibility for *S. aureus* (24 µg/mL) and no susceptibility for *E. coli* (Claeson *et al*., 1992). The MIC values for *S. aureus* (4 mg/mL) and *E. coli* (not susceptible at the highest concentration tested, 64mg/mL) noted in this study correlate with the results obtained by Claeson *et al*. (1992) and suggest that T-cadinol may in part be responsible for the antimicrobial activity noted in the *P. grandidentatus* oil.

The MIC results for *P. grandidentatus,* obtained when the volatile constituents were removed from the extract, indicate mostly increased activity. Only *E. faecalis* and *E. coli* demonstrated no susceptibility in the non-volatile extract. These pathogens also displayed poor to no antimicrobial activity when exposed to the oils.

When fresh plant material was extracted containing both volatile and non-volatile constituents increased efficacy was only noted for *E. faecalis.* As in the case with most microbial species studied against *T. camphoratus*, the dried combined plant material indicated higher antimicrobial activities with four pathogens (*S. aureus, E. faecalis, P. aeruginosa* and *C. neoformans*), displaying increased efficacies when volatiles and non-volatiles were combined. Thus only the dried combined plant sample containing volatile and non-volatile constituents were selected to comparatively investigate the essential oil (volatile) and non-volatile extract (Figure 9.12). When observing the ethnobotanical use of the species, no correlation could be found for the preferred use of dried material over fresh material and a review of the *Plectranthus* genus by Lukhoba *et al.* (2006), when reviewing 62 species, make no reference to the use of fresh or dried plant material.

The isobologram ratio study for *S. aureus* show predominantly synergistic profiles for seven of the nine ratios studied (Figure 9.13). The two ratios expressing antagonism is where the extract is at highest concentrations, (80:20%) and the ratio 90:10%. The point is not seen on isobologram as the ratio is greater than the scale expressed. It is interesting to note than when observing the MIC data (Table 9.6) the essential oil (4 mg/mL) has far less efficacy than the non-volatiles devoid of oil (0.5 mg/mL), yet when combined, efficacy is enhanced both in the isobologram study and with the MIC study (DC).

Enterococcus faecalis indicated the highest synergistic effect when observing both the fresh combined material and dried combined material combination (Table 9.6). This enhanced

synergistic pattern was corroborated in the isobologram study where synergy was noted for all ratios investigated (Figure 9.13).

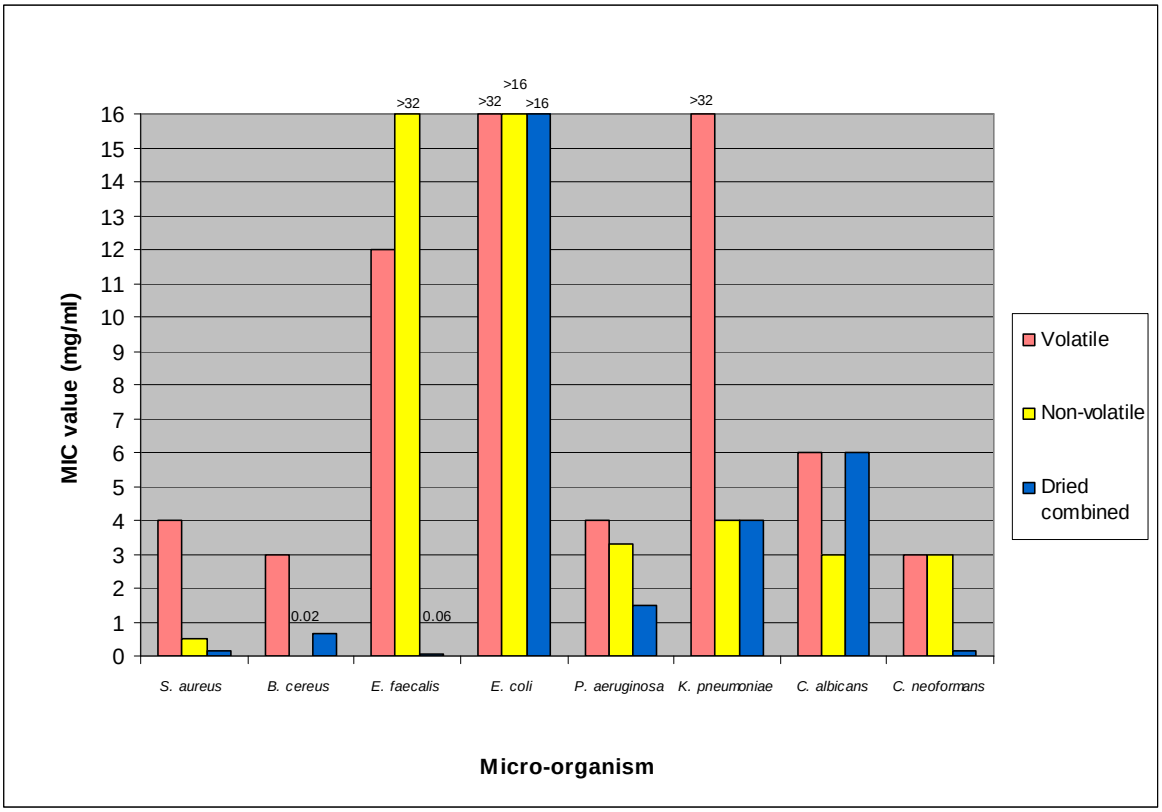


Figure 9.12 The comparative MIC (mg/L) for the volatile constituents (EO), non-volatile constituents (NV) and dried combined (DC) constituents for *P. grandidentatus.*

This pattern was also noted for the isobologram ratio study for *K. pneumoniae* (Figure 9.13) and MIC data (Table 9.6), however, to a lesser extent. The dried combined plant material having volatile and non-volatile constituents displayed a MIC value of 4 mg/mL while the essential oil exhibited no activity. All ratios within the isobologram study for *K. pneumoniae* displayed a synergistic pattern.

All 1:1 ratios on the isobolograms (depicted as squares), show synergism. Due to the close proximity of the ratio points for the *K. pneumoniae* and *E. faecalis* isobologram no square was inserted as this would impair data visualization.

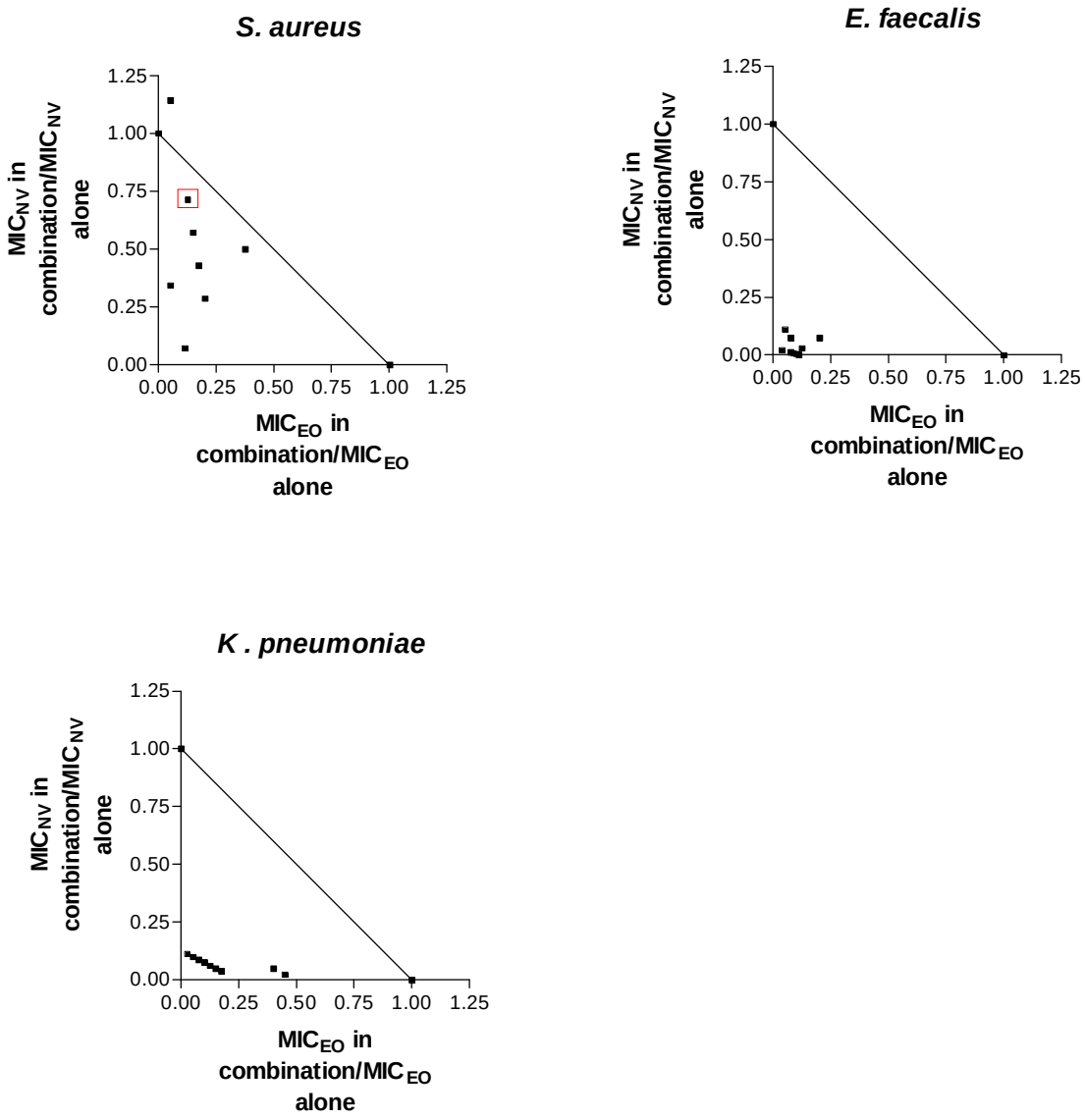


Figure 9.13 The antimicrobial efficacy of the test organisms *S. aureus, K. pneumoniae* and *E. faecalis* respectively against varying concentrations of the non-volatile (NV) and volatile (EO) constituents of *P. grandidentatus.*

From this work it is shown that there may be considerable differences in microbiological activity between volatiles obtained by distillation and non-volatiles obtain through extraction. The greatest variation seen in the study on *T. camphoratus* is where *E. coli* indicated an MIC value of 16.0 mg/mL for the essential oil and the non-volatile plant extract had a MIC value of 3.0 mg/mL, thus exhibiting more than a 5-fold difference. Similarly for *P. grandidentatus* where *B. cereus* has a MIC value of 3.0 mg/mL for the essential oil and the non-volatile plant extract had a MIC value of 0.02 mg/mL. When plant material comprised of both volatile and non-volatile constituents, efficacy was enhanced for most pathogens. Increased efficacies were predominantly

found for the dried combined plant material over the fresh material. Eloff (1998b), affirmed that the choice of dried over fresh plant material for biological studies were favoured by a number of researchers.

In a similar study (Lalli, 2006), undertaken on three *Pelargonium* species, the comparative TLC and HPLC data were included and indicated no decomposition of non-volatile compounds during distillation. Confirmation was also made using TLC to ensure that the plant material remaining in the distillation apparatus (NV) contained no essential oil constituents.

Even though the ratio combination of the volatile / non-volatile mixtures used in this study does not truly reflect the natural ratios in the whole plant, it is clear that synergistic mechanisms exist, which necessitates the inclusion of volatile compounds in the antimicrobial assessment. One also needs to consider the dosage and administration of herbal medicines when observing targeted activity. The various different administration methods (infusions, inhalations, tinctures, deconcoctions etc.) are usually carefully selected by the healer for the greatest efficacy, where preparation often serves to neutralize toxins (van Wyk *et al.*, 1997). It has also been demonstrated (Bonati, 1980) that the tincture of *Valeriana* is more potent than the preparation in water. The administration of an infused oil, where the oil acts as a penetrative enhancer for active principles of the extract is also widely accepted (Shealy, 1998) and conceivably the combination of volatile / non-volatile constituents improve solubility and bioactivity of the active principles.

The actual mode of action where the essential oil and non-volatiles interact to enhance activity is complex and may be attributed to a number of different mechanisms. It is known that essential oils disrupt the bacterial cell wall (Burt, 2004) and a number of studies have confirmed that aromatic compounds exert their antimicrobial efficacy on the cytoplasmic membrane by altering its structure and function (Cox *et al.*, 2000; Lambert *et al.*, 2001; Holley and Patel, 2005). This was clearly observed with confocal membrane integrity studies undertaken on *O. asteriscoides* (Chapter 3.6.2), however, the antimicrobial effects of essential oils may be dependent on a number of other factors such as pathogen specificity, oil concentration, level of activity and relevant function of the microbial membrane such as pH gradient, ATPase activity, electrochemical potential, ion leakage, changes in composition of fatty acid and intracellular absorption of material (Nychas *et al.*, 2002). Such complexities require further investigation, however, one could postulate that the essential oils provide a different mode of action to the non-

volatiles and thus act synergistically to enhance activity. This synergistic interaction could possibly enhance activities in a similar manner to other combined antimicrobials such as amoxicillin and clavulanic acid. Here the mechanism of action is known, where clavulanic acid while independently exerts little antimicrobial activity, in combination with amoxicillin, increases the spectrum of activity by inactivating ß-lactamases, blocking active enzyme sites and thus allowing for a greater efficacy (Gibbon, 2005). Similarly essential oils could enhance entry into the cell and allow for selected non-volatile components to exert bactericidal activity.

9.7 General conclusions

- The major constituents of the essential oil of *Tarchonanthus camphoratus* are β-caryophyllene (13.4%) and to a lesser extent 1,8-cineole (9.3%), representing 22.7% accumulatively. The two major compounds in the essential oil of *Plectranthus grandidentatus* are T-cadinol (10.3%) and camphor (15.2%), representing 25.5% accumulatively.

- The non-volatile constituents devoid of any essential oils show higher antimicrobial efficacy than the volatile constituents. The only exceptions were *Cryptococcus neoformans* and *Enterococcus faecalis* when exposed to *Tarchonanthus camphoratus* and *Plectranthus grandidentatus* respectively.

- When volatile constituents were combined with non-volatile constituents (fresh and dried plant material), MIC values most often indicated better efficacy for the dried combined (volatile and non-volatile) plant material.

- Isobologram representation of the combination of various ratios of essential oil and non-volatile extracts devoid of essential oils, present a predominant synergistic profile for all pathogens for *Tarchonanthus camphoratus* and *Plectranthus grandidentatus*.

- The correlation between MIC data and isobolograms were mainly congruent where the greatest synergy for *Tarchonanthus camphoratus* was noted for *Bacillus cereus, Klebsiella pneumoniae Candida albicans* and *Cryptococcus neoformans*. The greatest synergy for *Plectranthus grandidentatus* was observed with *Enterococcus faecalis.* These efficacies

correlate with the traditional use of the plants to treat stomach ailments and respiratory conditions.

Chapter 10

A comparative investigation of the antimicrobial properties of indigenous South African aromatic plants with popular commercially available essential oils.

10.1 Introduction

Popular commercial oils, such as lavender (*Lavendula angustifolia*), thyme (*Thymus vulgaris*), tea tree (*Melaleuca alternifolia*), peppermint (*Mentha piperita*) and rosemary (*Rosmarinus officinalis*) have been used extensively to treat bacterial and fungal infections associated with the urinary tract, wounds, skin disorders, digestion, as well as the respiratory tract (Shealy, 1998). Their popular use for antimicrobially related disorders validated the choice to comparatively investigate these commercial essential oils with five indigenous South African essential oils used in ethnic healing rites. While literature (Carson and Riley, 1995; Mangena and Muyima, 1999; Tassou *et al.*, 2000 Angioni *et al.*, 2004) documents and supports the antimicrobial action of these commercial oils, their popularity has been complimented by excellent marketing strategies. Even though the majority of the rural South African population consult traditional practitioners, for urban dwellers there is still a trend to rather use globally recognized herbal therapies, of which popular essential oils is considered the most widely used of all natural therapies (Nakatsu *et al*., 2000). Based on traditional use, consultation with literature and results obtained from research, five indigenous aromatic plants were selected from this thesis for comparative study: *M. flabellifolius* (Chapter 2), *O. asteriscoides* (Chapter 3), *A. afra* (Chapter 4), *L. javanica* (Chapter 5) and *H. natalensis* (Chapter 8). While antimicrobial activities have been assessed for these indigenous oils, this study aims to directly compare, under identical laboratory conditions, the antimicrobial activity against the five commercial oils mentioned above. The objective was to demonstrate the potential to actively promote South African indigenous aromatic plants and their essential oils for antimicrobial therapy.

10.2 Botanical description and distribution

An abridged botanical description with distribution map for indigenous oils can be found in the relevant corresponding Chapters. For the commercial plant oils:

Lavendula angustifolia, commonly known as lavender is a member of the Lamiaceae family. It is native to the Mediterranean but is widely cultivated throughout Europe. This bushy evergreen shrub bears grey leaves turning greener with age. Most commonly blue to mauve-coloured flowers (Figure 10.1) are borne in the summer months (Lis-Balchin, 2002).

Thymus vulgaris or thyme as it is commonly known, is also from the Lamiaceae family. It is a small hardy shrub perennial to the Mediterranean. Leaves are dark grey to green in colour-bearing small white or violet flowers (Figure 10.2) which bloom from spring to summer (van Wyk and Wink, 2004).

Melaleuca alternifolia or tea tree, is a member of the Myrtaceae family, indigenous to Australia. The evergreen tree grows in swampy, low-lying areas having needle-like leaves (Figure 10.3). Stalkless, yellow or purple flowers are borne in the flowering season (Shealy, 1998).

Rosmarinus officinalis (rosemary), from the Lamiaceae family is native to the Mediterranean, but cultivated world-wide for its culinary, perfumery and medicinal uses. It is a woody perennial shrub with evergreen spiky leaves. The flowers are mainly purple in colour but may vary between pale pink to blue (Figure 10.4). This highly aromatic herb has many traditional uses and was often planted in cemeteries where the odour emitted was thought to stimulate memories of deceased loved ones (Shealy, 1998).

Mentha piperita, a perennial mint plant from the Lamiaceae family, originates from Europe and is well-known as a flavouring and therapeutic agent. The use of the plant dates back to 300 BC where hieroglyphics show the use of mint as an ingredient in incense. Historical anecdotes have shown that the Greeks and Romans have used mint at their feasts and the Arabs have prepared mint in beverages. Of the many species of mint available, *Mentha piperita* is regarded as therapeutically more superior due to the high menthol content. The copious amount of menthol produced is a characteristic feature of the plant, giving it the distinctive peppermint odour. The

leaves are short with finely-toothed margins (Figure 10.5) producing spiked pink to mauve flowers in late summer (Wootton, 2005).



Figure 10.1 *Lavendula angustifolia* (lavender)



Figure 10.2 *Thymus vulgaris* (thyme)



Figure 10.3 *Melaleuca alternifolia* (tea tree)[*1]



Figure 10.4 *Rosmarinus officinalis* (rosemary)



Figure 10.5 *Mentha piperita* (peppermint) [*2]

[*1] http://yourskin101.com/natural-skin-care/tea-tree-oil.aspx; [*2] http://sophy.u-3mrs.fr/photo-id/Men

10.3 Medicinal uses

A description of the medicinal uses for indigenous oils can be found in the relevant corresponding Chapters. For the commercial plant oils:

Lavendula angustifolia: The essential oil from this species is one of the most popular and appreciated in phytotherapy (Lis-Balchin, 2002). The name of the plant is derived from the Latin word “lavare” meaning “to wash” referring to the medicinal use for the washing of wounds. The medicinal properties range widely from the treatment of coughs and colds by steam inhalation to applying topically for the treatment of burns and acne (Shealy, 1998).

Thymus vulgaris: The essential oil from this species was traditionally used as a medicine for treating coughs and respiratory disorders associated with the upper respiratory tract. It is known as an antiseptic to treat wounds and urinary tract infections (Shealy, 1998). There have been a number of reports validating the antimicrobial efficacy of thyme (Delespaul *et al*., 2000; Valero and Salmerón, 2003) and in a publication by Lai and Roy (2004), where the antimicrobial activity is reviewed by a number of authors against bacterial isolates such as *S. aureus*, *B. subtilis* and *E. coli*, in conjunction with other organisms. Due to its known antimicrobial efficacy it is often used in combination with other oils as a preservative.

Melaleuca alternifolia: The Australian aborigines have originally used tea tree oil as an antiseptic and the leaves were used as a poultice for skin infections. It is a popular choice for the effective treatment of respiratory complaints where a few drops of the oil are placed into hot water and the vapours inhaled. Diluted tea tree oil is used as a mouthwash to protect against dental caries. The demand for tea tree oil as a disinfectant increased when it was used by Australian soldiers participating in World War I (http://www.vitacost.com/science/hn/Herb/Tea_ Tree.htm). The use of tea tree oil to treat topical fungal infections (with particular reference to *Candida albicans*) is well-known. Several *in vitro* studies have revealed that the oil inhibits many strains of bacteria, yeasts and fungi (Carson and Riley, 1995; Shealy, 1998; Caelli *et al*., 2000; Cox *et al*., 2001).

Rosmarinus officinalis: The oil is known for its antiseptic and disinfectant properties (Shealy, 1998). The use of rosemary dates back to the ancient Egyptians where it was used in cleansing rituals. In former times this aromatic herb was rubbed on uncooked meat to delay putrefaction. During outbreaks of the plague, rosemary was a common ingredient used in posies carried to

ward off infection. More recently rosemary is used in the fumigation of French hospitals. Of the many virtues bestowed on rosemary, the therapeutic value has been accredited to the treatment of bronchitis, sinusitis, as an expectorant, as a mucolytic and antiseptic. The therapeutic application by inhalation has also been used by both aromatherapists and traditional Chinese medicinal practitioners. Furthermore, it has been used as a scalp treatment for the eradication of dandruff (Holmes, 1999; Wootton, 2005).

Mentha piperita: The medicinal use of the oil has been used to treat sinusitis by inhalation therapy. It has been used for the treatment of skin disorders but is mostly known for digestive disorders, hence the traditional consumption of peppermints after a large meal (Shealy, 1998). In addition to the numerous reports on the antimicrobial properties of mint, as cited by Logan and Beaulne (2002), the use of enteric coated peppermint oil was found to be beneficial in the treatment of overgrowth of intestinal flora, often associated with irritable bowel syndrome.

10.4 Methods

10.4.1 Chemical aspects

Essential oils: The aerial parts of the five indigenous plants were collected from various localities within South Africa and hydrodistilled for three hours in a Clevenger apparatus according to methods described in Chapter 2.5.1. Plant samples were retained for identification and voucher specimens housed within the Department of Pharmacy and Pharmacology at the University of the Witwatersrand. The corresponding voucher numbers are given in Table 10.1. The five commercial essential oils with their technical data were obtained from Robertet (France) where distillation and analysis was carried out according to ISO compliance.

Gas chromatography (GC) and gas chromatography combined with mass spectrometry (GC-MS): Even though the GC and GC-MS analysis of the indigenous oils have been previously undertaken, all ten essential oils and the standards were confirmed on a Shimadzu 17A gas chromatograph using the following parameters: Column: J&W-DB-1 (30 m x 0.25 mm, 0.25 µm film thickness), Temperatures: injection port 230 °C, column 60 °C for 1 min., 5 °C / min to 180 °C, 180 °C for 2 min., (total = 25 min.). Quantification was achieved by correlation of relative retention times with authentic standards. The major compounds and their relative percentages are presented in Table 10.1 and Figure 10.6.

10.4.2 Antimicrobial aspects

The culture preparation was undertaken according to the NCCLS (2003) guidelines (Chapter 2.5.2) and the microplate MIC bioassay method was used (Carson *et al*., 1995; Eloff, 1998a; NCCLS, 2003), as in previous Chapters. Tests were performed in triplicate and identical laboratory conditions were maintained to ensure that inoculum, batch media, preparative techniques and incubation times were standardized. Where MIC data indicated coinciding values between commercial and indigenous oils, the MIC was further refined to narrow the increments. This was achieved by altering the dilution factor by preparing consecutive dilutions of increments 0.75, 0.72 and 0.65 (depending on the predetermined MIC). The MIC assay is performed on the principle of doubling dilutions with a ratio of 0.5 for each dilution. By manipulating the dilution factor i.e. changing the ratio, a narrower MIC value can be obtained. This would enable one to obtain a MIC reading somewhere between the two doubling dilutions. This can be demonstrated by observing the *Myrothamnus flabellifolius* oil sample, initially having an MIC value of 8 mg/mL when using the MIC doubling dilution principle. By changing the dilution ratio to 0.72, a MIC reading of either 16.59; 11.94; 8.60 (actual value obtained); 6.19 or 4.46 could be obtained, thus reducing the doubling dilution error normally encountered in MIC methodologies.

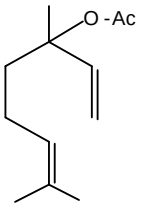
Time-kill studies: To comparatively demonstrate the time-kill efficacy, commercial and indigenous oils were exposed to *S. aureus*, *K. pneumoniae* and *C. albicans* (Figures 10.7-10.9) under identical laboratory conditions and the cidal efficacy plotted over time against the logarithm of viable colonies. The assays were carried out in duplicate according to methodology described in Chapter 2.5.2. Only one oil concentration (0.5%; 5.2 mg/mL) for all ten test samples was comparatively evaluated.

10.5 Results and discussion

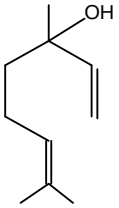
10.5.1 Essential oil chemistry

The major compounds in the commercial oils, identified by gas chromatography are presented in Figure 10.6. This ensured that commercial oils were of a high quality and gave some indication of which indigenous chemotype was included in this study. The major compounds for the indigenous oils have been diagrammatically presented in previous Chapters. A summary of data for all oils comparatively evaluated can be observed in Table 10.1.

Lavendula angustifolia

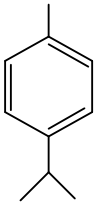


linalyl acetate

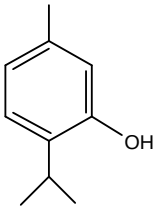


linalool

Thymus vulgaris

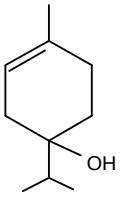


p-cymene

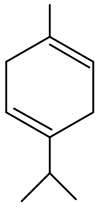


thymol

Melaleuca alternifolia

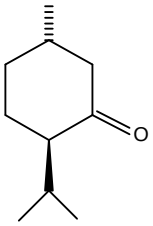


terpinen-4-ol

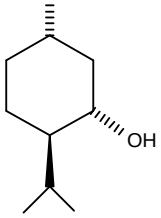


γ-terpinene

Mentha piperita

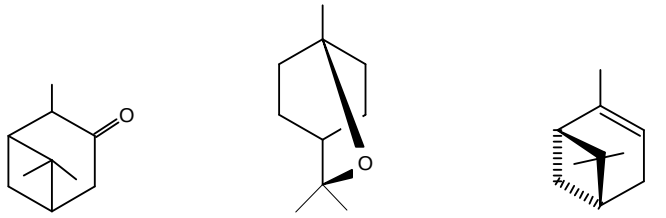


menthone



menthol

Rosmarinus officinalis



camphor 1,8-cineole α-pinene

Figure 10.6 Chemical structures for the major compounds identified in the commercial oils of *L. angustifolia, T. vulgaris, M. alternifolia, M. piperita* and *R. officinalis.*

Table 10.1 A summary of the major compounds and therapeutic use for the five indigenous and five commercial essential oils.

Species, family and voucher number	**Common name**	**Major compounds and relative %**	**Therapeutic use**
Lavendula angustifolia (Lamiaceae) Commercial sample	Lavender	linalyl acetate (31.3%), linalool (30.8%)	Acne, skin abrasions and wounds, antiseptic, respiratory tract infections.
Thymus vulgaris (Lamiaceae) Commercial sample	Thyme	thymol (47.2%), *p*-cymene (22.1%)	Wounds, antiseptic, urinary tract infections, colds, coughs and flu.
Melaleuca alternifolia (Myrtaceae) Commercial sample	Tea Tree	Terpinen-4-ol (38.6%), γ-terpinene (21.6%)	Acne, skin abrasions and wounds, antiseptic, respiratory tract infections, fever, fungal infections.
Mentha piperita (Lamiaceae) Commercial sample	Peppermint	menthone (18.2%), menthol (42.9 %)	Mainly for digestive disorders, respiratory ailments, skin lesions and acne.
Rosmarinus officinalis (Lamiaceae) Commercial sample	Rosemary	camphor (12.4%), 1,8-cineole (41.4%), α-pinene (13.3%)	Antiseptic and disinfectant.
Lippia javanica (Verbenaceae) Voucher number: AMV331	Fever Tea	β-ocimene (19%), linalool (65%)	Respiratory tract infections, fever, digestive disorders.
Artemisia afra (Asteraceae) Voucher number: AdcAV173	African Wormwood	1,8-cineole (17.8%), camphor (8.2%), α-thujone (18.7%), β-thujone (12.5%), artemisia ketone (10.1%)	Respiratory tract infections, fever, digestive disorders.

Species, family and voucher number	Common name	Major compounds and relative %	Therapeutic use
Heteropyxis natalensis (Heteropyxidaceae) Voucher number: AMV771	Lavender Tree	1,8-cineole (31.4%), limonene (17.9%), α-terpineol (7.3%)	Respiratory tract infections.
Osmitopsis asteriscoides (Asteraceae) Voucher number: ADCAV174	'Bels'	1,8-cineole (56%), (-)-camphor (14.8%)	Respiratory tract infections, inflammation, skin lesions, digestive disorders, fever.
Myrothamnus flabellifolius (Myrothamnaceae) Voucher number: AV169	Resurrection Bush	pinocarvone (11.1 %) *trans*-pinocarveol (19.6%) limonene (6.1%) *trans-p*-mentha-1(7), 8-dien 2-ol (7.4%) *cis*-p-mentha-1(7), 8-dien 2-ol (6.9%)	Respiratory tract infections, skin abrasions and wounds, urinary tract infections, mouth ulcers.

10.5.2 Antimicrobial activity

Initial MIC data indicated that indigenous essential oils compare favourably with the commercial oils as noted for *S. aureus* where *M. flabellifolius* and *O. asteriscoides* (4 mg/mL) showed higher sensitivity than the commercial oils: *L. angustifolia* (lavender), *M. alternifolia* (tea tree), *M. piperita* (peppermint) and *R. officinalis* (rosemary) at 8 mg/mL. However, in many instances there was very little variance between MIC values for indigenous and commercial oils. This was noticed for *B. cereus* where *O. asteriscoides, H. natalensis, T. vulgaris* (thyme), *M. alternifolia* (tea tree), *M. piperita* (peppermint) and *R. officinalis* (rosemary) oils where the same MIC value (4 mg/mL) was recorded (not shown in Table 10.2). A narrower MIC was hence determined. The final refined MIC values are depicted in Table 10.2. Minimum inhibitory values against all ten pathogens generally ranged between 4-16 mg/mL for most oils. A few exceptions are seen e.g. with the indigenous oils *M. flabellifolius* indicating the highest sensitivity towards *B. cereus* (2 mg/mL) and *S. epidermidis* (3.2 mg/mL). This was also noted for *O. asteriscoides* (1 mg/mL) against *C. neoformans.* Similarly, *T. vulgaris* (thyme) oil showed the highest sensitivity towards *S. aureus* (1.3 mg/mL) and *E. coli* (0.5 mg/mL). Previous comparative studies (Hammer *et al.*, 1999) also support high antimicrobial activity for *T. vulgaris* (thyme) where the lowest MIC reported was against *E. coli* (0.03%), in comparison with oils from 20 other genera.

Table 10.2 The MIC (mg/mL) of the five indigenous and five commercial oils.

Pathogen	***Lippia javanica***	***Artemisia afra***	***Heteropyxis natalensis***	***Osmitopsis asteriscoides***	***Myrothamnus flabellifolius***	***Lavendula angustifolia* (Lavender)**	***Thymus vulgaris* (Thyme)**	***Melaleuca alternifolia* (Tea Tree)**	***Mentha piperita* (Peppermint)**	***Rosmarinus officinalis* (Rosemary)**	**Control***
Staphylococcus aureus ATCC 12600	16.0	4.5	8.6	4.0	4.0	8.6	1.3	8.6	11.9	6.2	1.0×10^{-3}
Staphylococcus epidermidis ATCC 2223	7.6	16.0	5.7	6.2	3.2	6.2	4.7	6.2	6.2	10.1	2.5×10^{-3}
Bacillus cereus ATCC 11778	16.0	11.9	4.5	3.7	2.0	11.9	8.8	2.4	3.7	3.7	1.0×10^{-3}
Bacillus subtilis ATCC 6051	32.0	32.0	32.0	6.2	8.6	32.0	11.9	6.2	4.5	32.0	2.5×10^{-3}
Escherichia coli ATCC 11775	1.6	8.6	4.5	3.7	5.7	6.2	0.5	3.7	5.7	4.5	0.1×10^{-3}
Pseudomonas aeruginosa ATCC 9027	16.0	8.6	8.6	8.6	8.6	8.6	8.6	8.6	8.6	6.2	2.5×10^{-3}
Enterococcus faecalis ATCC 29212	13.5	5.7	18.0	7.0	6.2	18.0	4.0	8.6	6.2	24.0	2.0×10^{-4}
Klebsiella pneumoniae NCTC 9633	7.6	10.1	10.1	11.9	5.7	16.0	4.0	4.0	6.2	16.0	1.3×10^{-3}
Candida albicans ATCC 10231	8.6	4.5	4.5	5.7	5.7	5.7	2.4	3.7	2.4	5.7	1.3×10^{-3}
Cryptococcus neoformans ATCC 90112	2.4	8.0	5.7	1.0	2.0	4.0	4.0	4.0	4.0	1.6	2.5×10^{-3}

***The bacterial control was ciprofloxacin and fungal control was amphotericin B.

Results of these studies encompassing all five of the commercial oils were mostly congruent with previous findings. Carson and Riley (1995), confirmed results obtained for tea tree oil where it was also observed that *E. coli* was more susceptible than *S. aureus.* In another study, where eleven oils were comparatively evaluated for antibacterial activity, thyme oil showed a much higher inhibitory action than peppermint oil against fifteen of the eighteen pathogens studied (Özkan *et al*., 2003). Similarly in this study thyme showed higher inhibitory action in eight out of ten pathogens investigated. While MIC data for some of these oils have been reported, studies are not always directly comparable due to different methods, microbial sensitivities etc., hence the data represented here can be accurately compared due to the standardization of test conditions. *Thymus vulgaris* (thyme) oil showed the highest overall antimicrobial activity in the MIC assay followed by *M. flabellifolius* and then *O. asteriscoides* and *M. alternifolia* (tea tree) oil.

Time-kill data for *S. aureus* (Figure 10.7) indicate that of the ten oils studied, three of the four having bactericidal activity are from indigenous South African plants. Indigenous oils *O. asteriscoides* indicated bactericidal efficacy at 24 hr and both *M. flabellifolius* and *A. afra* displayed a cidal effect at four hours. *Thymus vulgaris* (thyme) was the only commercial oil showing bactericidal efficacy (at eight hours). These results showed some correlation with the MIC data where the same four oils had the lowest MIC values for *S. aureus.* The remaining six oils (*L. angustifolia*, *R. officinalis*, *M. alternifolia*, *M. piperita*, *H. natalensis* and *L. javanica*) show very similar bacteriostatic patterns, averaging approximately 50% reduction in CFU / mL after 24 hr.

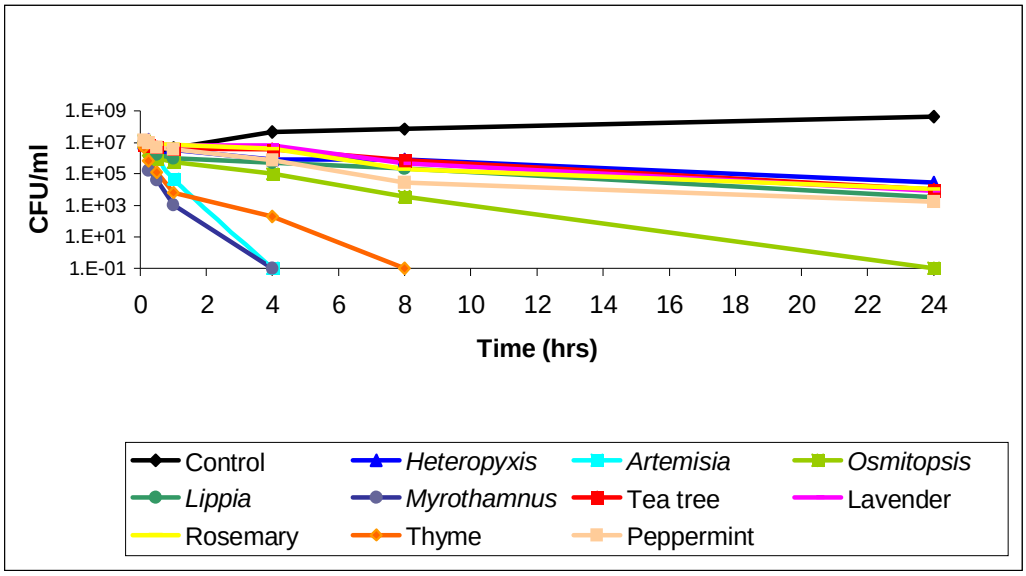


Figure 10.7 A comparative death kinetic study of *S. aureus*, exposed to 0.5% essential oil from five commercial and five indigenous plants over a 24 hr period.

Death kinetics for *K. pneumoniae* (Figure 10.8) indicated less sensitivity. Although a rapid reduction of CFU was observed within one hour for *O. asteriscoides, A. afra* and *M. piperita* (peppermint) oil, regrowth of *K. pneumoniae* was evident within one hour for *A. afra* and *M. piperita* (peppermint) oil and more gradually (after four hours) with *O. asteriscoides*. Bactericidal efficacy was noted for *T. vulgaris* (thyme) oil, *M. alternifolia* (tea tree) oil, as well as *M. flabellifolius* oil, having a time-kill effect within one hour. Again these results confirm similar susceptibility patterns as seen in the MIC data where the same three oils have the lowest MIC values for *K. pneumoniae*.

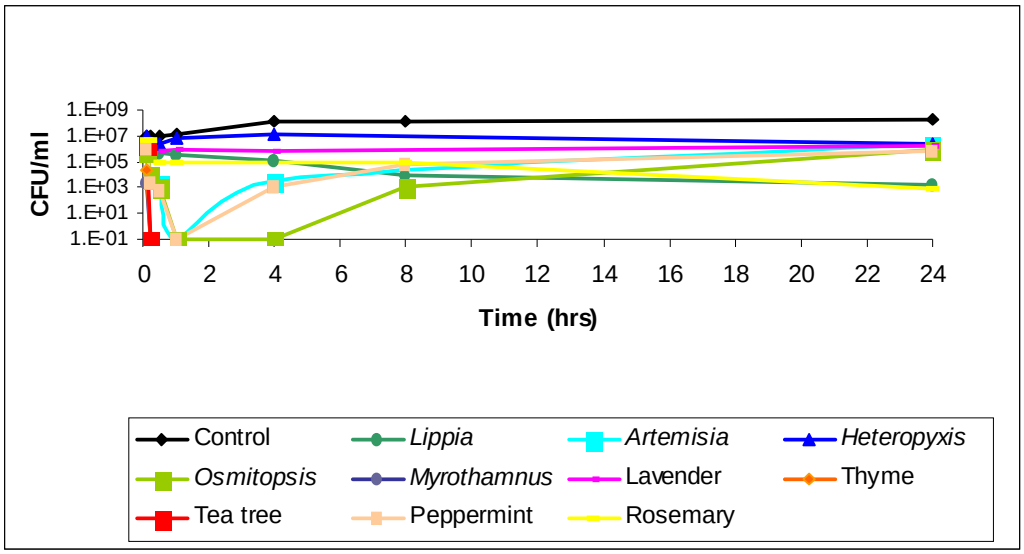


Figure 10.8 A comparative death kinetic study of *K. pneumoniae,* exposed to 0.5% essential oil from five commercial and five indigenous plants over a 24 hr period.

Most of the oils showed fungicidal efficacy for *C. albicans* (Figure 10.9) with the exception of *L. angustifolia* (lavender) and *H. natalensis* having limited static activity. Even though an approximate 25% reduction of CFU was noted for these two oils, no cidal activity was observed after the 24 hr test period. The oils of *O. asteriscoides, A. afra* and *T. vulgaris* (thyme) indicated time-kill death rates within four hours. The oil *M. flabellifolius* exhibited the most rapid (within one hour) bactericidal activity against *C. albicans.* Surprisingly *M. alternifolia* (tea tree) oil, marketed for it's anti-candidal activity did not indicate cidal efficacy in the first four hours but only after 24 hr, with oil samples *R. officinalis* (rosemary), *M. piperita* (peppermint) and *L. javanica*. This reduced time-kill effect was also noted in a comparative study (Hammer *et al.*, 1999) in which the same five commercial oils, amongst others, were studied. Results were

mainly congruent with *M. alternifolia* (tea tree) oil being less effective than *T. vulgaris* (thyme) and *L. angustifolia* (lavender) oils for *C. albicans*. Of the four oils demonstrating cidal efficacy for *C. albicans* within four hours, three (*M. flabellifolius, O. asteriscoides* and *A. afra*) are attributed to the indigenous oils.

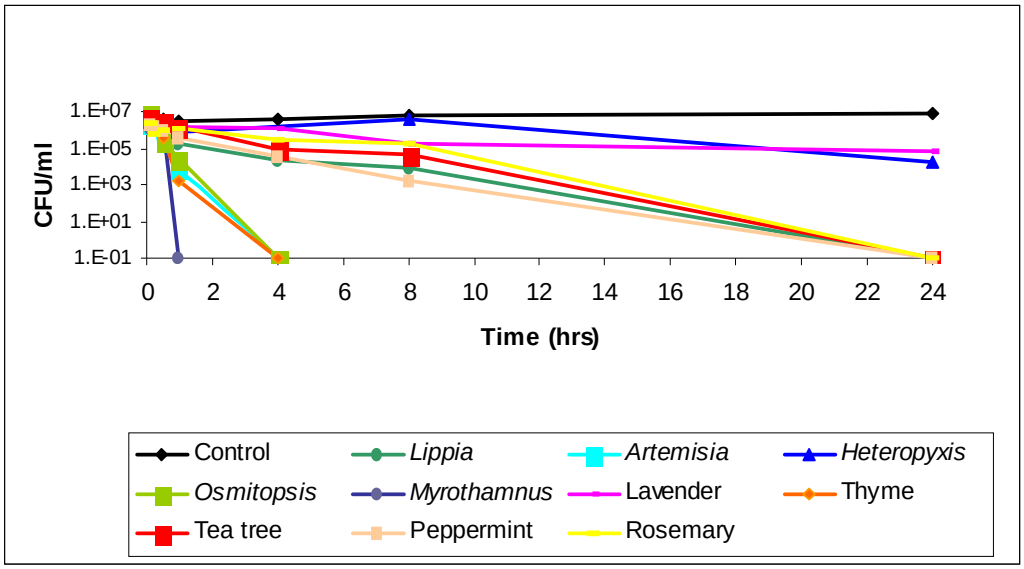


Figure 10.9 A comparative death kinetic study of *C. albicans*, exposed to 0.5% essential oil from five commercial and five indigenous plants over a 24 hr period.

In light of these findings we can confirm that the indigenous oils compared most favourably with commercial oils and as indicated in the death kinetic assay *M. flabellifolius*, an indigenous oil had the most rapid cidal effect for all three pathogens *S. aureus, K. pneumoniae* and *C. albicans*. Earlier studies (Chapter 2, Viljoen *et al*., 2002) state that the major compound (*trans*-pinocarveol, 19.6%) in *M. flabellifolius* is also used in pharmaceutical preparations such as Ozopulmin™, thus further substantiating the plant's potential for medicinal use. Comparative death kinetics (Lattaoui and Tantaoui-Elaraki, 1994) for the oils of three *Thymus* species (*T. broussonettii, T. zygis* and *T. satureioides*) had time-kill efficacies within three and a half hours against *S. aureus* (0.4% oil concentration) and *C. albicans* (0.2% oil concentration). While *T. vulgaris* (thyme) oil used in this study showed the highest microbial efficacies of all five commercial oils studied at 0.5% concentrations, the death kinetic rate was four hours and eight hours for *C. albicans* and *S. aureus* respectively. From both these studies, it can be confirmed that some *Thymus* species possess strong antimicrobial properties. In a time-kill study by May *et al*. (2000), the rapid bactericidal effect of *M. alternifolia* (tea tree) oil within 30 min was also

noted, confirming the time-kill data presented in this study. A time-kill study of *M. alternifolia* oil (Christoph and Stahl-Biskup, 2001) indicated a very similar cidal trend against *S. aureus* to that seen in this study, however, the authors demonstrated that combinations with other oils, such as manuka, showed excellent bactericidal efficacy, thus one should not exclude synergistic oil combinations in microbial therapy.

In summary these results validate the use of South African aromatic plants for their anti-infective properties and emphasize the need to promote the natural botanical resources in South Africa. Many urban South African people are growing European herbs for medicinal purposes but are reluctant or ignorant of the use of African plants for therapeutic use (Derwent, 2004). Thus, by publicising the efficacy of indigenous oils in comparison with those that are internationally well marketed (van Vuuren and Viljoen, 2006), a bridge may possibly be formed between western herbal practices and that of indigenous sources that have been known for centuries within the ethnic people.

We need to take into account however, that while these *in vitro* antimicrobial results support the local traditional use of indigenous aromatic plants, harvesting limitations need to be considered to make further commercial development a viable proposition. Also, of note is that not all *in vitro* antimicrobial results correlate with their therapeutic use and other factors such as chemophysical, pharmacological or physiological aspects may contribute to further enhance the efficacy of these essential oils.

10.6 General conclusions

- Comparative MIC results for popular commercial oils (*Lavendula angustifolia, Thymus vulgaris, Melaleuca alternifolia, Mentha piperita* and *Rosmarinus officinalis*) against the essential oils of five indigenous plants (*Myrothamnus flabellifolius, Osmitopsis asteriscoides, Heteropyxis natalensis, Artemisia afra* and *Lippia javanica*) indicated little variance for many samples with MIC values mainly ranging between 4-16 mg/mL.

- Time-kill data for *Staphylococcus aureus* indicate that of the ten oils studied, three of the four having bactericidal activity are from indigenous South African plants i.e. *Osmitopsis asteriscoides, Myrothamnus flabellifolius* and *Artemisia afra* with *Thymus vulgaris* as the only commercial oil showing bactericidal efficacy.

- Death kinetics for *Klebsiella pneumoniae* indicated less sensitivity, with bactericidal efficacy noted for *Thymus vulgaris*, *Melaleuca alternifolia* and *Myrothamnus flabellifolius* oil, having a time-kill effect within one hour.

- Eight of the ten oils investigated demonstrated fungicidal efficacy for *Candida albicans* in the time-kill assay.

- Of all oils studied, *Myrothamnus flabellifolius* showed the most rapid cidal effect against all three pathogens tested.

- Results indicate that the antimicrobial activity of South African indigenous essential oils compare favourably with the commercial oils investigated in this study.

Chapter 11

A review of the methods used to study the antimicrobial activity of aromatic plants.

11.1 Introduction

Many papers on the antimicrobial efficacy of plant essential oils and extracts have been published, however, there still remains a number of disparities in the techniques, where there is a need to explore standardization (Rios and Recio, 2005). This is evident even though numerous publications have been dedicated to the methods involved in bio-activity studies of extracts and oils (Remmal and Tanaoui-Elaraki, 1993; Brantner *et al*., 1994; Carson *et al*., 1995; Pauli and Kubeczka, 1997; Janssen *et al*., 1987; Eloff, 1998a; Marston and Hostettmann, 1999; Lahlou, 2004; Rios and Recio, 2005). As microbiological methods incorporate living principles (test micro-organisms), predictability of the outcome is not always clear and subject to many environmental influences that may impact on a response. Hence, the problems encountered when trying to standardize techniques. Standardization of protocols for antimicrobial testing of plants has limitations since the study objectives and experimental design amongst researchers often differ (Holley and Patel, 2005).

The methodologies involved in the empirical approach to discover biological activities should be carefully considered and approached so that results yield accurate and reproducible outcomes (Marston and Hostettmann, 1999). It is of the utmost importance that parameters such as plant collection, validation of laboratory equipment, chemical analysis and various intricacies of antimicrobial investigations are carefully defined. This Chapter serves to document the standardization of techniques based on literature reviews and practical suggestions when performing microbiological assays.

Microbiological assays on natural products are techniques where the potency of a test substance (plant extract, essential oil or compound) may be determined against the growth of a micro-organism.

The classification of such studies can be four-fold. There should be an initial screening process. Ideally this should be relatively cost effective, quick and need not be quantitative (Marston and Hostettmann, 1999). In these microbiological studies, disc diffusion assays meet these requirements. Secondly, screening should follow which confirms qualitative biological activity (Marston and Hostettmann, 1999). This has been achieved by determining the quantitative concentration that will inhibit microbial growth using the minimum inhibitory concentration (MIC) method. Both disc diffusion and MIC techniques may be carried out with the aid of automated equipment, however, all assays undertaken in this thesis have been carried out manually. Thirdly, monitoring assays should be in place for studies, such as isolation of antimicrobial constituents from whole plants, as in the case of bioassay-guided fractionation procedures. Secondary testing should be in place after monitoring studies to confirm activity, specifically for studies involving the isolation of active compounds. Hostettmann (1999) recommended that biological assays be used in conjunction with chemical screening to allow for a multidisciplinary approach that may yield important information for future drug development. Monitoring assays also include time-kill studies where viability is assessed over a selected time period. Lastly, a more outcome based approach may be undertaken to study the activity in more detail, as with synergy studies where a specific outcome is sought to test a hypothesis (Marston and Hostettmann, 1999).

11.2 Plant collection and essential oil isolation

For all plant collections voucher specimens were established and species correctly identified in order to botanically authenticate the plant material. For many follow-up studies plant material was re-collected, always from the same locality to avoid any chemotypic variability. There are several methods that can be used to isolate the essential oils i.e. cold press extraction, extraction of a single oil with another, steam or hydrodistillation, simultaneous distillation coupled with solvent extraction, supercritical fluid extraction, microwave extraction, solid phase extraction and fluorocarbon extraction (Nakatsu *et al.*, 2000). For these studies isolation was standardized by hydrodistillation as described in Chapter 2.2. This is the most common and best suited method for the isolation of volatile constituents in plant-based studies (Guenther, 1948). As recommended, distilled oils were stored at 4 ºC, in airtight amber vials to prevent alteration of composition (Burt, 2004). Traceability of oil samples to voucher numbers and oil yields were documented in an established reference book.

11.3 Equipment

All equipment i.e. from laminar flow benches, balances to micropipettes were calibrated according to standard operating procedures. This is imperative for good laboratory technique to ensure accuracy and reproducibility of results. Documentation supporting this is housed within the Department of Pharmacy and Pharmacology, University of Witwatersrand.

11.4 Chemical evaluation of essential oils

A review paper (Lahlou, 2004) on techniques used in the investigation of the bioactivity of essential oils lists the analytical requirements that should be adhered to when performing investigations on essential oils. Parameters such as equipment specifications, column type and dimension, carrier gas-flow rate, temperature of column and detector should be specified. Specifications for these studies have been documented in Chapter 2.5.1.

11.5 Effect of solvents, media selection and additions

A number of different solvents may be used for initial sample dilution preparation. The choice of an adequate biological solvent is crucial and examples include methanol, acetone, dimethyl sulfoxide (DMSO), and ethanol, amongst others. As many of these solvents are antimicrobial in nature they should be avoided if possible. Unfortunately studies on certain extracts show poor solubility in acetone as was the case with the *T. camphoratus* study (Chapter 9). Therefore a solvent such as DMSO which has a broad polarity range was used to facilitate dissolution of extract. For such studies, controls should be run of the solvent independently to determine the antimicrobial end points of the solvent. This was undertaken in the *T. camphoratus* study and DMSO showed inhibition between 4-8 mg/mL depending on the pathogen studied. Any antimicrobial activity within this range obtained with the *T. camphoratus* extracts were excluded, as activity could not be categorically attributed to the extract and may be as a result of the solvent. When investigating bio-activity, acetone should be used wherever possible as the solvent of choice, due to its inert properties and miscibility with water (Eloff, 1998b). The use of acetone for antimicrobial susceptibility testing is widely accepted as the solvent of choice and supported by over 444 references when performing a database literature search.

11.6 Reference standards

All assays must include conventional antimicrobial controls that are appraised under identical laboratory conditions to that of the assay. These confirm that the micro-organism is susceptible to the selected antimicrobial. In the literature review by Lahlou (2004), a number of references are cited where ciprofloxacin is recommended as one the ideal antibacterial controls and similarly amphotericin B is recommended for control testing against fungal organisms. Antimicrobial controls were assessed with every test procedure and evaluated against an acceptable standard (Chapter 2.5.2).

11.7 Pathogen selection

Many plants have reported use for the treatment of cuts, wounds, sores, ear infections, respiratory disorders, gastrointestinal upsets etc. indicating a possible bacterial or fungal infectious agent. In this current study the pathogen selection was based on the traditional use of the plant as determined from the ethnobotanical literature. Studies on the medicinal use of the plants were restricted to infectious test organisms. Even though a number of other pathogens exist, which may offer pertinent information for specific diseases, for this study a standard set of pathogens relevant to the ethnobotanical uses of the plant have been used. This would also allow for standardization of growth requirements. The culturing of many other pathogens involves either the addition of blood i.e. the *Streptococcus* species, provision of growth supplementation as in the case of *Mycobacterium* species and may even require enrichment of the incubation atmosphere with the addition of 5% CO_2 as in the case for *Lactobacillus* species. An overall pathogenesis of all the test organisms studied and their relative importance as infectious agents are given here so that pathogen selection based on the ethnobotanical use is clarified.

11.7.1 The Gram-positive test organisms

Staphylococcus aureus: Even though an opportunistic pathogen residing in the upper respiratory tract, *S. aureus* is one of the most virulent bacterial pathogens known to man-kind. The organism is responsible for superficial skin diseases (pustules, boils, carbuncles, abscesses, pimples and impetigo), systemic infections such as food poisoning and respiratory diseases causing pneumonia. This organism is commonly associated with bloodstream infections responsible for endocarditis, soft tissue infection and osteomyelitis (Rubin *et al*., 1999). A review of nosocomial

cardiac infections (Giamarellou, 2002) cites *S. aureus* as one of the major causative pathogens of bacteraemia responsible for 52-57% infections. The rate of increase of such infections has increased exponentially since 1980, escalating by 122% to 283%, depending on the hospital studied. With the ever increasing use of conventional antimicrobials such as methicillin (Hanberger *et al*., 2001) and vancomycin (Gibbons, 2004), resistance has emerged requiring a more aggressive search for newer anti-staphylococcal etiological agents. The emergence of methicillin resistant *S. aureus* (MRSA) strains have become a global problem where at certain epidemiology surveillance centres it is now referred to epidemic methicillin resistant *S. aureus* (EMRSA). Acquisition of MRSA carries increased healthcare costs and seriously limits effective antimicrobial therapy (Bannister *et al*., 2000). With the unsuccessful attempts to control multiresistant strains of *S. aureus* with conventional antimicrobials, it is no wonder that the search for newer antimicrobial sources from natural products has become increasing popular.

Staphylococcus epidermidis: Of the many infections transmitted, those entering via the skin usually occur due to natural immune mechanisms becoming suppressed or through abrasions to the skin barrier. The most common infections are primary and associated with localized inflammation and invasion of the pathogen, however, because of the skin's contact with the environment, it is usually the first port of entry where infections are spread systematically throughout the body. *Staphylococcus epidermidis* is commonly isolated from the skin but also resides in the upper respiratory tract, lower intestine, urethra and vagina. Although commensal, recent medical invasive therapies (such as the insertion of intravascular cannulae) have allowed this organism to enter the blood stream of immunocompromised patients, resulting in the organism becoming pathogenic (Bannister *et al*., 2000). In a study on nosocomial infections (Giamarellou, 2002), *S. epidermidis* was featured together with *S. aureus* as the most common infectious agent isolated from ICU patients. It is well-known that *S. aureus* is resistant to a number of conventional antimicrobials and it is only a matter of time before we see the same thing occurring with *S. epidermidis*.

Bacillus cereus: Not only is this organism associated predominantly with gastrointestinal infections but there have also been reports of ocular infections, infections from wounds, pneumonia and infections in the urinary tract (Kotiranta *et al*., 2000). An outbreak of a respiratory tract infection associated with a *B. cereus* strain has been documented by Gray *et al*. (1999), where the authors stated that such outbreaks are not uncommon. A review of the organism *B. cereus* classified infections into six groups i.e. local (including burns), bacteremia,

systemic (including meningitis), respiratory, endocarditis and gastrointestinal (Drobniewski, 1993). This spore bearing organism has been associated with an increasing number of food-borne diseases and given the ever present rise in such outbreaks, especially in rural areas where poor hygiene and a lack of adequate education is present, it would be ideal to use endemic plants as a prophylaxis to prevent such infections occurring.

Enterococcus faecalis: This pathogen is a regular inhabitant of the intestinal tract and often used as an indicator of faecal pollution. The species has been implicated in urinary tract infections, abdominal lesions and endocarditis (Boyd and Hoerl, 1981). In recent years the organism has emerged as a significant antibiotic resistant nosocomial pathogen, developing resistance to both aminoglycosides and vancomycin (Giamarellou, 2002). Vancomycin-resistant *E. faecalis* (VRE) has acquired specific resistant genes enabling the organism to withstand vigorous treatment protocols (Walsh and Amyes, 2004). Recently, the added concern that VRE strains could exchange genetic material with MRSA has resulted in pharmaceutical companies increasing their efforts to identify and produce compounds that would be active against these multi-resistant strains (Rice, 2006). Bannister *et al.* (2000), reports that over 25% of nosocomial infections resulting from surgery involving intravascular cannulae were attributed to *E. faecalis* species.

Bacillus subtilis: Even though rarely pathogenic, many ethnobotanical antimicrobial publications include this organism in their test organism data set, possibly due to the fact that this spore bearing Gram-positive organism may yield common inhibitory trends with other Gram-positive pathogens. Kalemba and Kunicka (2003) specified that this organism is one of the most common test strains when investigating antimicrobial efficacies against essential oils. There have been occasional reports of food poisoning associated with the toxins produced by *B. subtilis* (Bannister *et al*., 2000), but this is rare.

11.7.2 The Gram-negative test organisms

Klebsiella pneumoniae: Even though *K. pneumoniae* forms part of the commensal flora of the intestine, the organism becomes an infectious agent when inhabiting the upper respiratory tract causing pneumonia. The pathogen has been identified as one of the major causes of septicaemia in paediatric wards (Boyd and Hoerl, 1981). Fatality is as high as 90% in untreated cases and the severity was highlighted with the recent outbreak of *K. pneumoniae* infections in South African neonatal hospital wards where the deaths of nine babies were reported (Green and Rondganger,

2004). *Klebsiella* associated meningitis, endophthalmitis, cholangitis, endocarditis and urinary tract infections, even though uncommon have also been noted (Bannister *et al.*, 2000). The emerging antimicrobial resistance to ampicillin and amoxicillin shows the need to develop more resilient means of preventing *Klebsiella* nosocomial infections.

Pseudomonas aeruginosa: This organism can invade virtually any living tissue and is frequently responsible for a wide spectrum of severe infections (Hanberger *et al.*, 2001). The growth requirements for *P. aeruginosa* are minimal and thus often found in inappropriately stored water. This opportunistic pathogen is responsible for a vast number of nosocomial infections in particular that of burn patients, pneumonia, dermatitis, open wounds and otitis externa where a high degree of antibiotic resistance has been reported. The organism has been associated with nosocomial cardiac infections (Giamarellou, 2002) as well as infections of the lungs with a death mortality of over 80%. *Pseudomonas aeruginosa* can cause keretoconjunctivitis where infections have derived from non-sterile ophthalmic cleaning fluids (Bannister *et al.*, 2000). Multi-drug treatment regimens are often necessary to eradicate this organism. *Pseudomonas* species together with MRSA have been identified as the most prominent organisms that are rapidly becoming resistant to all existing antibiotic agents (Rice, 2003).

Moraxella catarrhalis: One of the most common bacterial agents responsible for acute sinusitis. This opportunistic pathogen may also be responsible for bronchitis and pneumonia in the lower respiratory tract (Laurans and Orfila, 1991). The incidences of *M. catarrhalis* infections have resulted in this organism being a well-established pathogen, presenting itself in a number of respiratory infections as well as septicaemia and meningitis. The organism resides in the upper respiratory tract and has become the most common bacterial species isolated from patients presented with laryngitis (Verduin *et al.*, 2002). Because this organism often co-invades with other infectious agents future treatment developments should include targeting poly-microbial infections.

Escherichia coli: While *E. coli* is a consistent resident of the small intestine, some strains are pathogenic, causing intestinal and urinary tract infections as well as neonatal meningitis. Most infections are usually food-borne or spread from person to person via the faecal-oral route (Bannister *et al.*, 2000). Their role in human pathogenesis has only recently been appreciated, due to the distinguishing difficulties between typing pathogenic and commensal strains. Antimicrobial resistance towards *E. coli* has already been reported worldwide. Enterotoxigenic *E. coli* is presently recognized as the primary cause of diarrhoea in infants and adults,

particularly associated with traveller's diarrhoea. A recent publication by Qadri *et al.* (2005), strongly recommends that serious efforts be required for management of *E. coli* infections. In addition, the widespread misuse of veterinary antimicrobials has resulted in the introduction of antibiotic resistant *E. coli* strains into the endogenous microflora of animals. Resistant genes are then transferred to human strains where resistance proliferates (von Baum and Marre, 2005).

Yersinia enterocolitica: Transmission occurs through contaminated food, milk and water where *Y. enterocolitica* will cause gastroenteritis accompanied by severe abdominal pain (Bannister *et al.*, 2000). Another means of transmission is the result of animal-to-human contact. The organism is highly acid resistant, contributing to its survival and virulence in the stomach (Koornhof *et al*., 1999). Infections arising from this organism may also cause enlarged lymph nodes, subcutaneous nodules and septicaemia, with fatality rates higher than 50% (Boyd and Hoerl, 1981).

Salmonella typhimurium: Infections associated with *Salmonella* are a global problem and it has been estimated that over 16 million cases are reported yearly (House *et al.*, 2001). Consummation of infected poultry is one of the most common sources of salmonellosis where up to 60% of all incidences occur. *Salmonella* food poisoning and colitis usually result from exposure and occasionally there have been reports of *Salmonella*-related infections in the bones, joints and soft tissue, indicating blood-borne infections (Bannister *et al*., 2000). Disease is spread by person to person contact via the faecal-oral route. Many countries have experienced an unprecedented rise in salmonellosis and in South Africa, nosocomial death mortality is on the rise due to outbreaks of *S. typhimurium* (personal communication: personnel from the Reference Centre for Enteric Disease, NHLS).

Proteus vulgaris: This commensal gastrointestinal organism is often associated with bladder infections where there is inappropriate urine release resulting in chronically stagnant bladder reservoirs (Bannister *et al*., 2000). The most frequently isolated human pathogen, *P. vulgaris*, is often acquired in hospitals where transfer has been associated with inadequate hygienic protocols in the manipulation of catheters (Boyd and Hoerl, 1981).

Serratia odorifera: This micro-organism is free-living and capable of causing sepsis and urinary tract infections. Since the emergence of antibiotic resistance *Serratia* spp. infections have become increasingly prevalent. The organism requires very little growth stimulant and has been

identified in hand-washing solutions from hospitals. The main route of infection is person to person contact with immunocompromised hospital patients (Boyd and Hoerl, 1981), however, the natural reservoir for *S. odorifera* is unknown (Lee *et al*., 2006). A fatal outbreak of *S. odorifera* occurred in neonatal wards in South Africa in the early nineties where contaminated parenteral intravenous solutions resulted in a number of infant deaths (Frean *et al*., 1994).

11.7.3 The fungal test organisms

Cryptococcus neoformans: This opportunistic pathogen can fatally infect those with compromised immune systems. Airborne spores enter the lungs where infection becomes asymptomatic until entry into the upper respiratory tract where pathogenesis is presented as *Cryptococcus* meningitis. Buchanan and Murphy (1998) cited a number of references supporting the emerging pathogenicity of *C. neoformans* resulting in 100% fatality for cryptococcal meningoencephalitis. Recently, *Cryptococcus* infections have become a problem in HIV patients (Bannister *et al*., 2000). In immunocompromised individuals, the infection may disseminate systemically causing infection of the central nervous system.

Candida albicans: This yeast is part of the normal microbial flora of the skin, mucosa and bowel. A fine balance with other flora is achieved in healthy individuals, however, during immunocompromised conditions or when antibiotic treatment is administered the balance is affected allowing for the growth proliferation of this organism. Moist skin exacerbates candidiasis resulting in superficial skin infections and inflammation at the site of infection. Cutaneous infection is commonly associated with nappy rash and genital infections (Bannister *et al*., 2000). Most candidal infections are effectively treated with antifungal agents such as nitroimidazoles, nevertheless the frequent use of potent antibiotics has often resulted in the over colonization of *C. albicans.* Over colonization often leads to mucosal infections of the mouth and throat, intestinal overgrowth and invasive persistent systemic infections. *Candida* related nosocomial infections have recently emerged and in a review by Giamarellou (2002), 73% of fungal infections resulting from prosthetic valve endocarditis were attributed to *Candida* spp.

Candida tropicalis: The number of reported infections associated with *Candida* species other than *albicans* has increased in incidence in the last two decades. In a report on *Candida* epidemiology surveillance, *C. tropicalis* was found in 16% of clinical *Candida* associated cases (Weinberger *et al*., 2005). In another study on candidemia patients, *C. tropicalis* was the third

most isolated *Candida* species isolated from ICU patients resident in Brazilian tertiary care hospitals (Colombo *et al*., 1999). The pathogeneses is similar to that of *C. albicans* and has been associated with nosocomial cardiac infections (Giamarellou, 2002).

Aspergillus niger: Aspergillosis infections usually reside in the external ear and lungs. Because of the characteristic fungal nature of spore formation and preference for damp conditions, this organism often resides in buildings and ventilation systems which may manifest in clinical respiratory infections associated with sick building syndrome. Lung infections may become difficult to treat, especially when colonizing immunosuppressed patients. If no response to the recommended treatment by amphotericin B occurs, surgery may become necessary to excise the infected lung segment (Bannister *et al*., 2000). Colonization of the organism in the cerumen of the ear may eventually lead to loss of hearing (Boyd and Hoerl, 1981).

Alternaria alternata: Although considered non-pathogenic, allergic responses are common when this organism has come into contact with the respiratory system. Airborne fungal spores enter the upper respiratory tract by inhalation where they may contribute towards allergic fungal sinusitis. *Alternaria alternata* spores are larger than other fungal spores and thus are more likely to be deposited within the mucus of the upper airway. A study by Shin *et al*. (2004), demonstrated a five fold increase in allergic immune response when exposed to *A. alternata*. The spores can also be a contributing factor in sick building syndrome.

11.8 Inoculum

For cultures, the strain number should always be specified (Paull and Marks, 1987; Janssen *et al*., 1987). In many papers, this has been neglected (Izzo *et al*., 1995; Rabe and van Staden, 1997; Natarajan *et al*., 2005). Wherever possible reference strains should ideally be used, as clinical strains, isolated from patients may have been exposed to conventional antimicrobials, thus having the potential to have devised a means of increasing resistance. The use of microbial culture reference standards has been standardized internationally. This allows for direct assay comparison whereas clinical strains are not universally available and could yield results that may differ from reference strains.

The importance of standardizing the inoculum size is discussed in a number of publications (Denyer and Hugo, 1991; Pauli and Kubeczka, 1997; Lambert, 2000). The general findings were that with the increase of inoculum size the MIC decreases. The recommended inoculum size for

assays testing antimicrobial agents should be standardized by visual inspection with a 0.5 McFarland suspension where the density is approximately equivalent to 1 x 10^{4-8} CFU/mL or alternatively adjusted photometrically (Klepser *et al.*, 1998; ESCMID, 2000; NCCLS 2003; Andrews, 2004). As the latter is based on turbidometric techniques, one must take into account that photometric readings cannot always predict viability of the test organism and even though readings may be standardized, if assays are not undertaken promptly, changes in broth density will occur due to exponential growth of the test organism.

In order to verify the standardization of inoculum when investigating biological activities of plant preparations, a selection of essential oils and commercial antibiotics were assessed with various different inoculum ranges. Eleven concentration ranges (0.1 – 30 mL) were prepared where a 24 hr growth of test organism (S. *aureus, K. pneumoniae* and *C. albicans*) was diluted with 100 mL Tryptone Soya broth. The MIC was determined for the two essential oil samples *O. asteriscoides* and *H. natalensis* (Table 11.1), as well as ciprofloxacin and amphotericin B (Table 11.2) where various inoculum sizes were introduced. Both, Table 11.1 and Table 11.2, summarise the MIC data obtained for the 11 concentration ranges as determined in triplicate and thus present the data as a range. It was interesting to note that the essential oils showed little variation in MIC value with increasing inoculum size and only slight variation was noted for the *H. natalensis* essential oil when investigating bacterial strains, however, with the yeast *C. albicans*, some variation was noted (Table 11.1). The MIC ranges for the conventional antimicrobials ciprofloxacin and amphotericin B indicated a substantial variation with increasing inoculum and thus extremely dependent on inoculum size (Table 11.2). This variation, although not predominantly noted with the essential oil samples, validates the need to standardize results and to keep in line with the literature recommendations. All assays undertaken in this thesis were thus adjusted according to the 0.5 McFarland standard.

Table 11.1 The MIC (mg/mL) for oil samples when tested with a varying inoculum density ranging from 0.1-30 mL:100 mL (24 hr culture: broth ratio).

Plant sample	**MIC range (mg/mL)**		
	S. aureus	***K. pneumoniae***	***C. albicans***
H. natalensis	8-16	8	8-32
O. asteriscoides	4	8	4-16

Table 11.2 The MIC (µg/mL) for controls when tested with a varying inoculum density ranging from 0.1-30 mL:100 mL (24 hr culture: broth ratio).

Control	MIC range (µg/mL)		
	S. aureus	***K. pneumoniae***	***C. albicans***
Ciprofloxacin	0.3-1.9	0.3-2.5	*
Amphotericin B	*	*	2.5-18.9

* Not tested, as amphotericin B is an inappropriate antimicrobial for the test organism.

11.9 Disc diffusion assay

The principle of disc diffusion assay is based on the outward diffusion of a growth inhibiting test substance into an agar base, which has been suitably inoculated with a pure culture of test organism. The plates are allowed to pre-diffuse at 4 °C for one hour allowing for dispersion of the test substance from the disc into the surrounding agar. Thereafter optimum incubation conditions follow which is predetermined depending on the selected test organism. Results are determined by measuring the clear zone of inhibition contrasting with the remainder of the agar having microbial growth (Hewitt and Vincent, 2003). For more detail of the method refer to Chapter 2.5.2.

A number of critical factors are involved in this assay and may affect the outcome. These are the following:

Measurement of the zone diameter: The simplest method of measurement is achieved with hand-held vernier callipers, where measurement is made to the nearest mm. The use of a light-box aids with visualization of zone size determination, however, in numerous publications much variation exists in obtaining an accurate measurement. For some publications (Hinou *et al*., 1989; Brantner and Grein, 1994; Kim *et al*., 1995; Mangena and Muyima, 1999), measurement of the inhibition zone includes the entire diameter including the disc (Figure 11.1). When no inhibition is noted, the result is presented as the size of the disc (6 mm). This is a relatively large area of "no-activity" and may become very difficult when comparing samples having slight activity i.e. 7/8 mm, as the actual inhibition is less than the size of the disc. In addition, one sees a negative result having a positive value. Other authors (Eksteen *et al*., 2001; Lourens *et al*., 2004; Njenga *et al*., 2005) make reference to only the distance from the disc edge to the area of

growth (Figure 11.2). This is a more accurate means of measurement, as the disc is not taken into account. It should be assumed that the active antimicrobial agents have diffused from the disc during the pre-diffusion period and thus should not be considered as an inhibitory area. Some publications, however, have not mentioned how the zone size has been determined (Akinpelu and Olorunmola, 2000; Dorman and Deans, 2000; Ezeifeka *et al*., 2004; Natarajan *et al*., 2005) and such discrepancies can greatly influence comparative literature evaluations. All disc diffusion assays undertaken in this thesis are standardized to measurements from disc edge to the area of microbial growth.

Choice of agar: The selection criteria for a growth medium for bioassays should be based on the nutrient requirements of the test organism. Pauli and Kubeczka (1997), in their overview of microbiological testing methods list 15 different media that have been used in the assessment of essential oils. A recent study (Moon *et al*., 2006) compared the efficacy of results achieved with two different media, IsoSensitest agar and Nutrient agar. No generalization concerning the superiority of one test medium over the other could be established. Mueller-Hinton media, however, is the recommended choice for bacterial assays (NCCLS, 2003). Both bacterial and fungal cultures were assayed in this thesis and the growth requirements for fungi differ from that of bacteria. As Mueller-Hinton media is not suitable for fungal culturing, Sabourauds Dextrose agar was selected. In later studies, to keep variables consistent, Tryptone Soya agar was used for both bacteria and fungi. In addition, this versatile medium with high nutrient value was selected due to its ability to produce a higher density of inoculum. In this way a more accurate definition between presence and absence of growth could be observed.



Figure 11.1



Figure 11.2

Figure 11.1 and 11.2 Agar with seeded test organism where the diameter of the zone of inhibition is measured (**Figure 11.1**) and where the radius of the zone of inhibition is measured (**Figure 11.2**).

Disc size and sample reservoir: The volume and concentration of test sample placed on the disc is important and should be consistent for all repetitions and between all test organisms (Hewitt and Vincent, 2003; Janssen *et al.*, 1987). Standardization of disc size to 6 mm has been undertaken in all disc diffusion studies and is recommended to ensure even absorption of test sample.

Diffusion: Even though disc diffusion methodology is a quick simple means of screening for antimicrobial activity, problems may arise associated with the hydrophobic nature of the samples assayed. Depending on the nature of the particular sample i.e. extracts and essential oils may differ and diffusion rates may result in inconsistent results. Essential oils do not easily diffuse through the agar and even with the pre-diffusion time allocation of one hour (Hewitt and Vincent, 2003), false negatives may still be encountered and the possibility of activity could be underestimated. Another prominent factor to be considered is the volatility of the oil samples. Depending on the incubation time a proportion of the oil is inclined to be lost due to evaporation and this too may impact on false negative results. This is particularly true for fungal test organisms due to their long incubation periods of 48 hr - 7 days (Janssen *et al*., 1987). It has been suggested (Southwell *et al*., 1993) that the hydrocarbon components are particularly prone to evaporation and thus one tends to evaluate antimicrobial activity against only a proportion of the essential oil compounds found in the whole essential oil sample.

Such false negative results may become problematic, particularly when comparatively evaluating methodologies. This was encountered in a number of instances in this thesis and is addressed further in Section 11.13 (Method variation).

11.10 Minimum inhibitory concentration (MIC)

The MIC measurement to determine antimicrobial activity is a quantitative method based on the principle of contact of a test organism to a series of dilutions of test substance. The assay determines the lowest concentration of antimicrobial agent that inhibits the growth of the test organism (Bannister *et al.*, 2000). Assays involving MIC methodology are widely used and an accepted criterion for measuring the susceptibility of organisms to inhibitors (Lambert and Pearson, 2000). Although current methods are straightforward, to obtain useful comparative information from tests is a challenge as factors such as inoculum size, incubation temperature, reading of results, growth medium and assay technique can greatly affect the results (Christofilogiannis, 2001). Three main references were used to carry out the MIC assays:

- The NCCLS (2003), used for obtaining an adequate accepted inoculum range.
- Eloff (1998a), suitable because the method has been refined for plant-based studies.
- Carson *et al.* (1995), suitable as the method takes into account essential oil volatility.

Due to the volatility of oil samples all microtitre plates were sealed with sterile adhesive sealing film. This ensured no loss of volatile actives. Inouye *et al.* (2001b) demonstrated the increased effect observed when sealing agar plates during agar dilution MIC assays. With this principle in mind, preliminary observations were undertaken on the MIC micro-well methodology where sterile sealing tape was placed on microtitre plates and comparatively evaluated with unsealed plates. Table 11.3 confirms the increased efficacy when the microtitre plate was sealed. With all three plant samples (*L. javanica*, *T. camphoratus* and *C. gratissimus* var. *subgratissimus*), where the essential oils and extracts were investigated, the MIC values were lowered with sealing. The results for ciprofloxacin (non-volatile) did not indicate any variation between sealed and unsealed areas.

Selection of growth medium: As with disc diffusion assays, Tryptone Soya medium was selected for most of the MIC studies as it is considered the most suitable medium for the growth of all test organisms. Studies on medium comparison were undertaken where the MIC's were performed in both Mueller-Hinton and Tryptone Soya broth under the same test conditions using the same test samples. Results indicated no difference in the outcome. Thus, to standardize procedures, it was decided to perform all MIC assays with Tryptone Soya medium.

Table 11.3 The mean MIC (mg/mL) for plant samples exposed to *K. pneumoniae* in microtitre plates that have been sealed in comparison to the uncovered microtitre plates.

Plant sample	Sample type	Uncovered (mg/mL)	Sealed (mg/mL)
L. javanica	Essential oil	16	12
	Extract	>32	6
T. camphoratus	Essential oil	>32	12
	Extract	12	4
C. gratissimus var. *sub-gratissimus*	Essential oil	>32	4
	Extract	>32	8
Ciprofloxacin control		0.2×10^{-3}	0.2×10^{-3}

***Reading of results*:** The use of tetrazolium salts as an indicator for biological activity has been successfully employed for many years (Begue and Kline, 1972). Its application for end point determination of MIC readings in natural product research has been widely accepted (Hamburger and Cordell, 1987; Brantner *et al.*, 1994; Eloff, 1998a). *p*-Iodonitrotetrazolium violet (INT) forms a red-purple dye on reduction i.e. in the presence of biological growth and thus when added to all wells within a microtitre plate, binds to living cells making end point MIC visualization more clear, however, in spite of the numerous references to microtitre MIC methods incorporating INT, no recommended time between the adding of indicator and reading of results could be clarified in literature. This factor is crucial as results may differ completely when read at different times. Practical experimentation demonstrated that if results were read within the first three hours of adding INT a much lower MIC value was obtained, however, this is not ideal, as the indicator salts have not had a full colour development and for this reason the reading of all MIC values was standardized at six hours after adding INT for bacterial test organisms. Continuous repetitions and replications confirmed that this time allocation resulted in a more reliable reading with full development of colour and minimum change of MIC. Ciprofloxacin did not show any variation in the MIC readings when observed periodically within the six hour colour development. For yeast cultures, an INT exposure time was increased to 24 hours as colour change developed at a much slower pace, possibly due to the delayed growth of yeasts. This was evident for both plant samples and the conventional antifungal control amphotericin B.

The reporting of MIC results for pharmacognosy-based studies differs somewhat from conventional MIC reports with known MIC breakpoints. To elaborate, one needs to understand how the clinician interprets data obtained from MIC testing. For each conventional antibiotic there is an acceptable breakpoint range which has been predetermined by susceptibility criteria and quality control (NCCLS, 2003). Thus MIC determinations on known antibiotics can be effectively compared to the recommended categories. Results are then categorized as either susceptible (an appropriate antimicrobial to treat infection), intermediate (where response may be lower) or resistant where the MIC values depict possibility of resistance arising. With pharmacognosy studies however, there are no comparative breakpoints as the antimicrobial activities are reported for test substances that have not been previously examined and used for therapeutic purposes. The activities found for plant-based assays are much higher then conventional antimicrobials. Activities of 8 mg/mL are considered acceptable (Fabry *et al.*, 1998) and many literature citations refer to natural products having activities over 1 mg/mL

(Gibbons, 2004). This is in contrast with commercial antimicrobials obtaining therapeutic susceptibilities in the lower µg range and should not be used as a direct comparison. The control antimicrobials are thus only included as a reference to monitor the response of the test organism to a known antimicrobial.

Much debate has arisen over the use of statistical methods, particularly standard deviation to report MIC results. Standard deviation is defined as the square root of variance and is used to compare the variance between samples within a data set (Roscoe, 1995). Such criteria are only useful and an acceptable means of measuring activity when a large number of samples are included in the data set and the variance between samples is narrow. With MIC reporting, the methodology incorporates doubling dilutions and therefore the variance, if reported as standard deviation becomes so high that even one variation i.e. results 16 to 32 mg/mL, are represented as a very high probable variance. The reporting of an MIC value is hence not a true absolute point and the true value is somewhere between the lowest test concentration that inhibits the test organism and the next lowest test concentration (Opalchenova and Obreshkova, 2003). For this reason many studies using MIC methodology do not report standard deviation. The use of standard deviation only becomes a valuable statistical tool when comparably evaluated to acceptable MIC limits given by the NCCLS (2003) quality control. Here the degree of variance can be reported, as direct comparisons can be made to the acceptable breakpoint limits. Even though the statistical method of using standard deviation was not used to analyze data, the experimental procedures should always be repeated to confirm data, as well as replicated at different times to ensure reproducibility, as was undertaken with the current studies.

***The use of a dispersing agent*:** Due to the oil volatility and problems associated with miscibility of the oil in an aqueous based medium, the use of Tween has been suggested to facilitate an emulsive mix of test substance. In Remmal and Tanaoui-Elaraki (1993) and Delespaul *et al.* (2000), the use of Tween resulted in increased MIC values. Inouye *et al.*, (2001b) reviewed a number of contradictory publications either supporting or rejecting the use of a dispersing agent in antimicrobial assessments. Practical application of the use of Tween (Saarchem) at 0.1-0.5% showed no difference in MIC values. To avoid interference of a dispersing agent on the biological system, the use of an emulsifier was excluded in the assays.

11.11 Bio-directed fractionation (bio-autographic assays)

The principle of the method is based on the isolation of the test substance (whole plant and / or active compound) by basic thin layer chromatography conditions (Chapter 6.5.1.1). The test compounds are then allowed to diffuse into a suitable agar medium containing a test micro-organism and incubated at optimum incubation temperatures. Antimicrobial compounds are thereafter detected by the lack of microbial growth in the vicinity of the test compound. Test compounds to be isolated are preferentially obtained from non-volatile constituents rather than essential oils, as the volatile constituents are often lost during incubation, leading to poor detection of inhibition. A number of variations of this method have been used to determine antimicrobial activity by target-directed isolation of antimicrobial compounds. The method most commonly used is where the selected test culture is mixed with suitable growth medium and sprayed onto a TLC plate. This was not selected for this study as aseptic conditions are difficult to maintain and with spraying, an aerosol of culture is created thus potentially contributing to contamination if multiple assays are being performed. This direct method also has the disadvantage of media drying out which would ultimately result in inconsistent inhibition zones. Similar inconsistencies would arise if the agar-culture spray mix is not of adequate uniform thickness.

The method best selected, having the least amount of variables, was the over layer method (Chapter 6.5.1.1). This method is applicable to a wide range of micro-organism, produces well defined zones of inhibition and is not susceptible to possible contamination (Marston and Hostettmann, 1999; Hostettmann, 1999).

11.12 Time-kill analysis

Time-kill studies provide descriptive information on the relationship between bactericidal activity and the concentration of test substance (Tam *et al*., 2005). Even though the methodology is labour intensive and requires a number of steps where variables may be introduced, the method gives valuable information of the cidal action over time. A number of considerations need to be taken into account when analyzing time-kill data. The type of organism studied may impact on the results observed. This was particularly noted for studies on *P. aeruginosa* where regrowth after initial reduction in CFU/mL was encountered. Previous studies have postulated that the regrowth phenomenon be attributed to preferential killing of sub-populations, however, this could not be substantiated in further studies as indicated by Tam *et al.* (2005) who attributed

regrowth more to the adaption of the test micro-organism to the environment containing test antimicrobial. Tam *et al.* (2005) stated that the constant exposure to a test antimicrobial may result in a more resistant mixture of bacterial populations which become increasingly difficult to kill over time. This substantiates results where there is an immediate reduction in CFU/mL over time followed by regrowth. This was noted in time-kill essential oil studies with *M. flabellifolius* (Chapter 2) and to a lesser extent *O. asteriscoides* (Chapter 3) when investigated with *P. aeruginosa*. This organism is known to produce multiple emerging resistant strains (Livermore, 2002) and time-kill studies have shown that even with conventional antimicrobials such as meropenem, regrowth is observed (Tam *et al.*, 2005). The regrowth effect may also be attributed to a concentration dependent dose, as noted in a death kinetic study on *C. gratissimus* var. *subgratissimus*. As limited essential oil was obtained, time-kill assays were employed for only one pathogen (*K. pneumoniae*). Selection was based on the moderate activity obtained in the MIC assay and the traditional use of *C. gratissimus* to treat coughs, colds and inflammation of the lungs. The essential oil, when exposed to *K. pneumoniae*, indicated initial bacteriostatic activity (Figure 11.3, not shown in Chapter 7) for all three concentrations (0.5%, 0.75% and 1%). The lowest concentration studied (0.5%) showed a 99.9% reduction in CFU within the first four hours, however, after eight hours regrowth occurred exponentially over the 24 hr test period. Both the 0.75% and 1% oil concentration indicated an immediate drop in CFU and within one hour bactericidal action was obtained and maintained for the full 24 hr test period. These results indicate that essential oil concentrations for *C. gratissimus* var. *subgratissimus* should be greater than 0.5% in order to achieve prolonged efficacy.

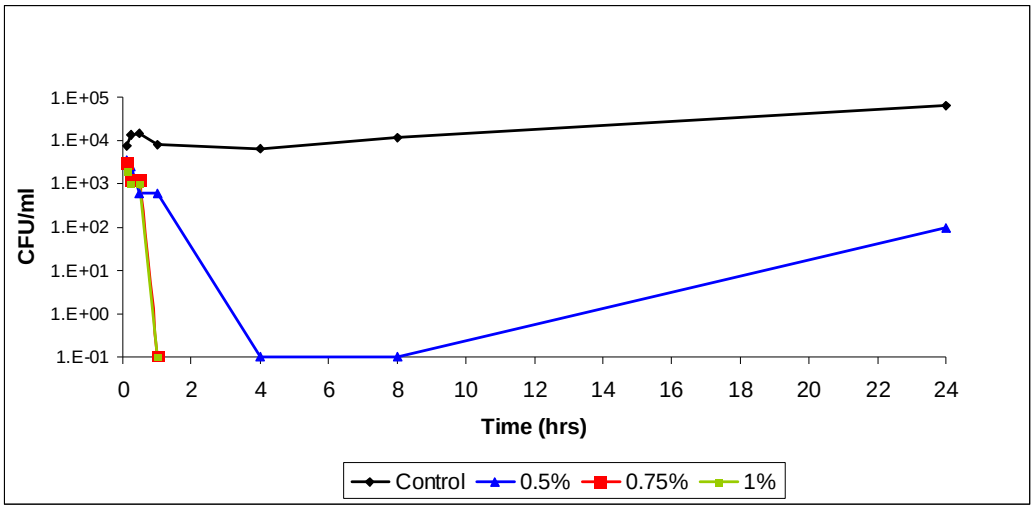


Figure 11.3 Death kinetic studies of *K. pneumoniae* exposed to the essential oil of *C. gratissimus* var. *subgratissimus* over 24 hr.

One must also take into consideration that *in vivo* pharmacodynamics of conventional antimicrobial drugs relies on repeated doses to achieve complete cidal activity. Should this principle be applied to time-kill analysis, one could extrapolate that a second dose provided with an extended time-kill may result in a cidal outcome.

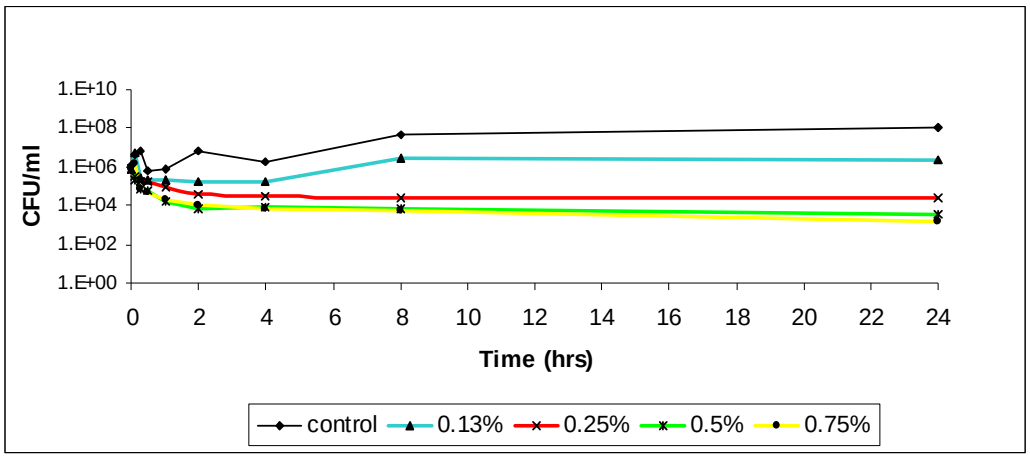


Figure 11.4 Death kinetic studies of *B. cereus* exposed to the essential oil of *P. grandidentatus* over 24 hr.

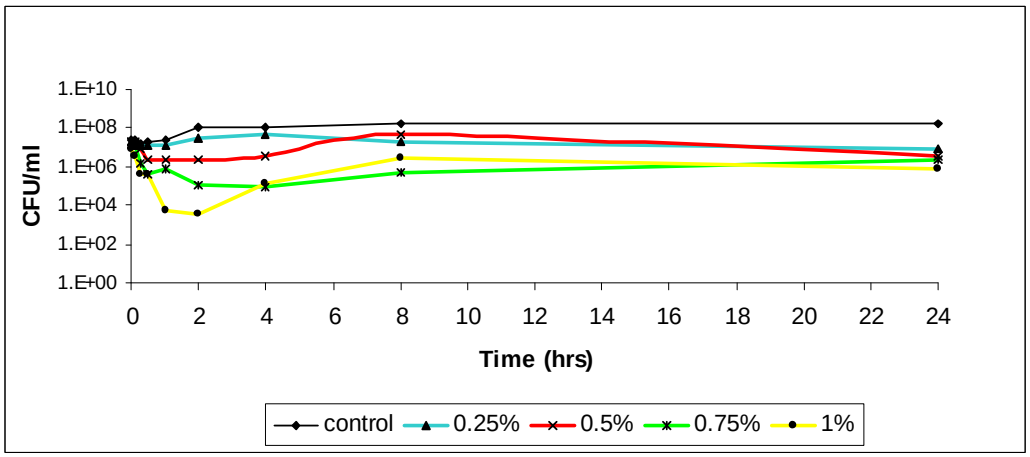


Figure 11.5 Death kinetic studies of *K. pneumoniae* exposed to the essential oil of *H. natalensis* over 24 hr.

The presence of spores may also affect the results. Bacterial spores are the most resistant of all cell forms to bacterial agents and only with vigorous destructive methods are spores inactivated. This was noted in the study on *P. grandidentatus*. The MIC data for *B. cereus* demonstrated

relatively high sensitivity (3 mg/mL) for the essential oil. Thus, a theoretical decision was made to perform a time-kill study to assess the pharmacodynamic effect over time. It came to light upon examination of the results (Figure 11.4) that the poor reduction of CFU/mL over time was due to the fact that this organism possesses spores which re-germinated in spite of the possibility of the vegetative cells been inactivated. Previous research on disinfectant evaluation with *B. cereus* has shown increased resistance of this species due to the presence of spores (Lindsay and von Holy, 1999; Lindsay *et al.*, 2002).

Even though time-kill studies are a valuable means of determining the inter-relationship between test essential oil and selected test pathogen, there are certain circumstances where studies may not yield informative data, as was the case with a study on a collective sample of *H. natalensis* essential oil where death kinetics performed on *K. pneumoniae* indicated little reduction in CFU/mL over time (Figure 11.5). These results do correlate with the moderate MIC data (seasonal mean of 8.8 mg/mL), but the time-kill assay gives a more detailed account of the pathogens immediate and prolonged response with exposure to the test oil.

11.13 Method variation

Although the antimicrobial activity of essential oils and their constituents are well-known (Yousef and Tawil, 1980; Hinou *et al.* 1989; Pattnaik *et al.* 1997; Hammer *et al.*, 1999), the methods used to assess such activity may result in varied reporting of results. In a number of studies, observed from both publications and determined through experimental procedures, variability between results was demonstrated.

The variability and problems associated with the disc diffusion assay have been well documented (Janssen *et al.*, 1987; Pauli and Kubeczka, 1997; Njenga *et al*., 2005; van Vuuren *et al*., 2006). Variation between disc diffusion results and MIC data was observed to a certain degree for all studies incorporating both methods. This was noted with the study on *A. afra* (Chapter 4) where lower disc diffusion efficacies were observed for three of the 13 pathogens when a comparative evaluation was made with the MIC methodology. The *L. javanica* study (Chapter 5) also incorporating both disc diffusion and MIC methodology indicated better efficacies with the MIC assay for five of the 12 pathogens studied. For *O. asteriscoides* (Chapter 3), results for four of the ten pathogens were incongruent. The greatest variation was noted for *B. subtilis* where no activity was noted in the disc diffusion assay and moderate activity (8 mg/mL) detected in the MIC assay. The variation of results noted using different methodologies was greatest with the *H.*

cymosum subsp. *cymosum* study (Chapter 6). All ten pathogens showed no activity for the essential oils in the disc diffusion assay and only Gram-positive sensitivity for the extracts, however, when the same samples were tested with the MIC method, all pathogens showed activity for the essential oils and the crude extracts were at least six times more active than the oil.

For a limited number of pathogens it was found that the disc diffusion methodology gave higher efficacies than the MIC methodology. This was evident for *A. afra* (Chapter 4) where *B. subtilis* and *C. tropicalis* had disc diffusion readings of 3 mm and 5 mm respectively. Less sensitive MIC values were obtained for *B. subtilis* (16 mg/mL with poor activity) and *C. tropicalis* (8 mg/mL with moderate activity). This was also noted for *L. javanica* where *E. faecalis* and *C. tropicalis* were more sensitive having disc diffusion values of 4 mm and 3 mm respectively and poor MIC values of 16 mg/mL and 32 mg/mL respectively. This was also noted in a study on *Eriocephalus* species (Njenga *et al*., 2005) where the disc diffusion results yielded better activities than MIC values.

It is often difficult to compare results obtained from MIC assays with literature presenting disc diffusion results only. This was noted in a number of studies such as results obtained for *A. afra* (Chapter 4) compared to the investigation by Graven *et al.* (1992). This was also noted in the *H. natalensis* (Chapter 8) study where correlating data with the non-quantitative results by Gundidza *et al.* (1993). When comparing results with literature, the latter trend seems to support the use of quantitative MIC methodology for pharmacognosy studies rather than earlier disc diffusion antimicrobial assessments. This is observed by the number of earlier publications using disc diffusion methods (Hinou *et al*., 1989; Graven *et al.,* 1992; Gundidza *et al.,* 1993; Brantner and Grein, 1994; Kim *et al*., 1995; Mangena and Muyima, 1999) and later publications utilizing MIC methods (Negi *et al*., 2005; Gaspar-Marques *et al*., 2006). The use of the more quantifiable MIC assay techniques are presently the preferred method of antimicrobial assessment (Kalemba and Kunicka, 2003; Burt, 2004).

11.14 Validation of various methodologies

As microbial cell viability is the key in determining antimicrobial activity, any reliable method of determining rate of growth or inhibition of growth can be used to assess antimicrobial activity. Diffusion assays, MIC's, UV methods and time-kill analysis are all such methods used to

confirm antimicrobial activity. It has been noted that different methods (Tassou *et al*., 2000), may not always be comparable, however, the ideal is to use reliable and reproducible techniques that can yield consistent information. It has been shown (Chapters 3, 6 and 8) that results from literature do not always correlate with experimental conditions. Standardization of techniques are thus valuable in order to provide a common basis for comparison.

This was achieved for time-kill assays, undertaken in two different laboratories to confirm reliability and consistency of results. Collaboration was undertaken with researchers at the University of Iowa whom have been involved with a number of studies reviewing the methodology of time-kill studies (Klepser *et al*., 1997; Klepser *et al*., 1998; Ernst *et al*., 1999). Time-kill assays were conducted using similar methods, same oil sample and same culture reference standards to determine the reliability and degree of variability. The results obtained for both comparative time-kill studies against *M. flabellifolius* (Viljoen *et al*., 2002) and *O. asteriscoides* (Viljoen *et al*., 2003) confirmed congruency. It was also shown how time-kill analysis and membrane integrity studies undertaken on *O. asteriscoides* correlated very well.

Congruency between disc diffusion, MIC and time-kill methods was examined for all relevant Chapters and the greatest correlation was noted in the study with *M. flabellifolius* (Chapter 2) where five pathogens showed consistent results between disc diffusion and MIC methods. Furthermore death kinetics of *S. aureus* and *P. aeruginosa* were congruent with both disc diffusion and MIC methods.

11.15 Interactive combination studies

The investigation of medicinal plants with a view to ascertain a single chemical entity for antimicrobial activity is becoming more and more improbable and research is now moving towards the investigation of a combination of substances to achieve efficacy. For plants to rely on a single compound in their biochemical warfare with pathogens would be equivalent to relying on the “single golden bullet” approach and thus as researchers investigating the activity of single compounds, we would be ignoring the evolutionary approach that plants may have developed various metabolic mechanisms for the production of structurally and functionally diverse compounds to overcome emerging resistance. To ensure future success in natural product research we must incorporate interactive phytochemical studies with existing practices in the hope that developments may be used as a foundation and driving force for novel

chemotherapeutic agents. The problem arises with the standardization of methods and even in the classification of interactive procedures (Williamson, 2001; Lambert *et al.*, 2003). The effect of combination studies may result in outcomes that are synergistic, antagonistic or additive. Synergism is evident where the combined action of test substances exceeds the effects of the individual components. Antagonism occurs where there is a reduced effect in combination. An additive effect occurs when the effect of the combination is equal to the sum of the components in combination (ESCMID, 2000). There are a number of different methodologies that can be utilized to express synergy or antagonism and different researchers have their preferred method of assessment. The time-kill method, has been praised as one of the best methodologies to study synergy (Beale and Sutherland, 1983) even though the methodology had been shunned by Berenbaum (1980). Further validation of the death kinetic method for synergistic studies was given by Acar (2000), where the method was praised from a clinical perspective. Time-kill studies have shown to be an effective procedure in demonstrating synergistic interactions between constituents of *O. asteriscoides* (Chapter 3, Viljoen *et al.*, 2003). In this study the combined effect of the two major constituents, camphor and 1,8-cineole, indicated enhanced efficacy for *C. albicans* when in combination. Similar studies were conducted on *A. afra* (Chapter 4, Viljoen *et al.*, 2006) where the major constituents, independently and in various combinations, were investigated to determine the role in the overall activity of the plant. Results indicate that all 11 samples (independently and in combination) when investigated at the concentration relative to that found in the whole plant, do not play a role in the overall activity of *A. afra*. In another study, time-kill methodology was used to validate the traditional use of *A. afra* when combined with *L. javanica* showing how the therapeutic properties may be enhanced when used in combination (Chapter 5).

An algebraic method to determine synergy by means of the fractional inhibitory concentration index (FIC) has been defined in Chapter 7.5.2 where the roots, stems and leaves of *C. gratissimus* var. *subgratissimus* were studied independently and in combination. Even though the definition and equation for determining the FIC is a widely accepted means of measuring interaction, the interpretation has evolved. Table 11.4 demonstrates the refinement of measuring the FIC index, with the most recent reference being the most concise FIC interpretation. It was therefore decided that the most comprehensive and up to date definition (Schelz *et al.*, 2006) would be used to classify synergy or antagonistic interactions.

Table 11.4 The classification of the FIC index in accordance with the corresponding authors.

Reference	Interaction			
	Synergy	**Additive**	**Indifference**	**Antagonism**
Kerry *et al*., 1975	≤0.7	*	*	≥1.3
Berenbaum, 1978	<1.0	1.0	*	>1.0
Krogstad and Moellering, 1986	≤0.5	*	1.0	≥2.0
ESCMID, 2000	≤0.5	>0.5-1.0	>1.0-<2.0	≥2.0
Mackay *et al.*, 2000; Odds, 2003	≤0.5	*	>0.5-≤4.0	>4.0
Schelz *et al*., 2006	≤0.5	>0.5-1.0	>1.0-≤4.0	>4.0

* Values not given by author

The isobole method of determining interaction is presently the favoured method of assessment (Williamson, 2001) and although complicated, gives a more accurate assessment of each agent when studied in various combinations. The method involves the combination of two samples at various ratios (Table 11.5), and determining the MIC value. The MIC for each sample in the concentration ratio is determined independently (Table 11.6). The MIC result for each sample in the combination is comparatively assessed against the MIC obtained for the independent sample and expressed as a dose ratio (Table 11.6). These ratios are then presented on an isobole graph. The adjoining line of the two axes (Figure 11.6) indicates the individual doses. It can be shown that one cannot always rely on a 1:1 combination and that depending on the ratio of the combination, differences in activity may be noted. This is demonstrated in a study where the volatile and non-volatile constituents of *T. camphoratus* were investigated with *E. coli.* Figure 11.6 in relation to Table 11.5 and Table 11.6 indicate where synergistic ratios were found (ratios 3-6, depicted in red). Antagonism was noted for ratio 8 (depicted in green) and an additive effect is noted for ratio 2 (depicted in brown). The novel isobologram representation of synergy thus presents three different interactions for the volatile and non-volatile constituents of *T. camphoratus* studied with *E. coli*, depending on the relative ratio in which the test compounds were combined.

Table 11.5 The relative ratios of the volatile and non-volatile constituents of *T. camphoratus.*

Samples	Ratio (%)										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Volatile	100	**90**	**80**	**70**	**60**	**50**	**40**	30	20	10	0
Non-volatile	0	**10**	**20**	**30**	**40**	**50**	**60**	70	80	90	100



Table 11.6 The raw data for the volatile and non-volatile constituents of *T. camphoratus.*

Row in plate	Concentration in mg/mL		MIC values		Ratio values	
	X (volatile)	**Y (non-volatile)**	**X (volatile)**	**Y (non-volatile)**	**X (volatile)**	**Y (non-volatile)**
1	16.0	0	8.00	0.00	1	0
2	**14.4**	**1.6**	**7.20**	**0.80**	**0.900**	**0.200**
3	**12.8**	**3.2**	**3.20**	**0.80**	**0.400**	**0.200**
4	**11.2**	**4.8**	**2.80**	**1.20**	**0.350**	**0.300**
5	**9.6**	**6.4**	**2.40**	**1.60**	**0.300**	**0.400**
6	**8.0**	**8.0**	**2.00**	**2.00**	**0.250**	**0.500**
7	**6.4**	**9.6**	**3.20**	**4.80**	**0.400**	**1.200**
8	4.8	11.2	2.40	5.60	0.300	1.400
9	3.2	12.8	1.60	6.40	0.200	1.600
10	1.6	14.4	0.80	7.20	0.100	1.800
11	0	16	0.00	4.00	0.000	1.000

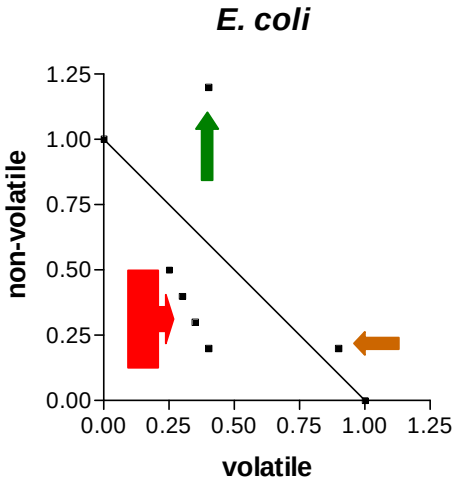


Figure 11.6 The isobole of the non-volatile constituents and volatile constituents combined in different ratios demonstrating the different profiles (antagonism, synergy and additive) dependent on the ratio studied (Table 11.6).

As seen with the comparison of disc diffusion and MIC methodologies, variation may also be noted when looking at different methods to assess synergistic and antagonistic interactions. This was highlighted by Odds (2003), wherein it was stated that reproducibility errors using MIC methodologies are common. Mackay *et al*., (2000) found good correlation of time-kill methods with calculation of FIC indices if methods were performed simultaneously. Up until recently the checkerboard MIC methodology and the calculation of FIC indices were the only methods adopted for synergy investigations and many studies (Ryan *et al.*, 1981; Lambert *et al.*, 2003) have focused on the correlation between the two. More recently e-tests have been incorporated in antimicrobial synergistic studies, however, these are only applicable to commercially available antimicrobials and have little relevance to plant-based studies.

The use of isobolograms for antimicrobial combinations has been poorly explored in spite of its wide acceptance in other interactive pharmacological studies, such as its application in drug interactions, antiparasitic studies, antiviral combinations, toxicology interactions and numerous *in vivo* investigations. A literature search yielded very few (Codd and Deasy, 1998; Cedergreen *et al.*, 2006; Kamatou *et al*., 2006) interactive antimicrobial studies using isobole methods. Recently, a mathematical 3-dimensional isobologram approach for the examination of antimicrobial agents was presented by Boucher and Tam (2006) validating isobologram construction as an effective tool for the examination of antimicrobial combinations. In the editorial by Odds (2003), researchers were encouraged to seek more sophisticated approaches to the measurement of synergy, to overcome limitations imposed by chequerboard methods. Thus interactive approaches should be further explored and validated with different assays to confirm interactions. With such validations in place, the justification and development of antimicrobial combinations could lead to patentable entities making research in the field of phytosynergy not only viable but also commercially beneficial (Lambert *et al*., 2003).

11.16 Summary

The standardization of antimicrobial assay techniques based on literature reviews and practical suggestions may be summed up briefly as follows:

- There should be suitable identification and documentation for all plant collections (Janssen *et al.*, 1987; Cos *et al*., 2006).

- Analytical GC-MS data for all oil constituents should be included in microbiological analysis to assess structure-activity relationships (Lahlou, 2004).

- Ideally, all microbial strains should belong to a standard collection i.e. ATCC (Cos *et al.*, 2006).

- More than one antimicrobial method should be undertaken in order to validate activity (Cos *et al*., 2006).

- Selection of the test micro-organism should be guided by the ethnobotanical use of the plant (Cos *et al.*, 2006).

- Standardization of inoculum should be undertaken for all assays (Pauli and Kubeczka, 1997; Cos *et al*., 2006).

- Inclusion of appropriate controls in each test replicate i.e. conventional antimicrobials and test blank should be incorporated with all assays (Cos *et al*., 2006).

- The occurrence of pharmacological interactions should be explored (Burt, 2004; Verpoorte *et al.*, 2005; Cos *et al*., 2006).

Chapter 12

General discussion, conclusion and further recommendations.

12.1 General discussion

Antimicrobial pharmacological activities of aromatic plants consider many different parameters, depending on the focus of the study. The studies presented herewith have demonstrated a number of factors that should be considered when performing antimicrobial plant-based studies. Moreover, essential oil studies are mplex due to the heterogeneous nature of the oil. Components not only exhibit distinct molecular formula, have specific physiochemical properties but also interact synergistically or antagonistically within the mixture (Lahlou, 2004). This thesis has thus followed a structure-activity related approach into the antimicrobial study of some of the most widely used aromatic medicinal plants used for the treatment of infectious diseases.

12.2 Thesis summary

Nine indigenous medicinal aromatic plants: *M. flabellifolius, O. asteriscoides, A. afra, L. javanica, H. cymosum* subsp. *cymosum, C. gratissimus* var. *subgratissimus, H. natalensis, T. camphoratus* and *P. grandidentatus* have been selected to investigate their essential oil composition and biological activity, establishing scientific evidence for their local use in herbal preparations. Figure 12.1 outlines the thesis in brief indicating plants studied, methods used and outcomes. A more extensive discussion follows based on the objectives listed in Chapter 1.

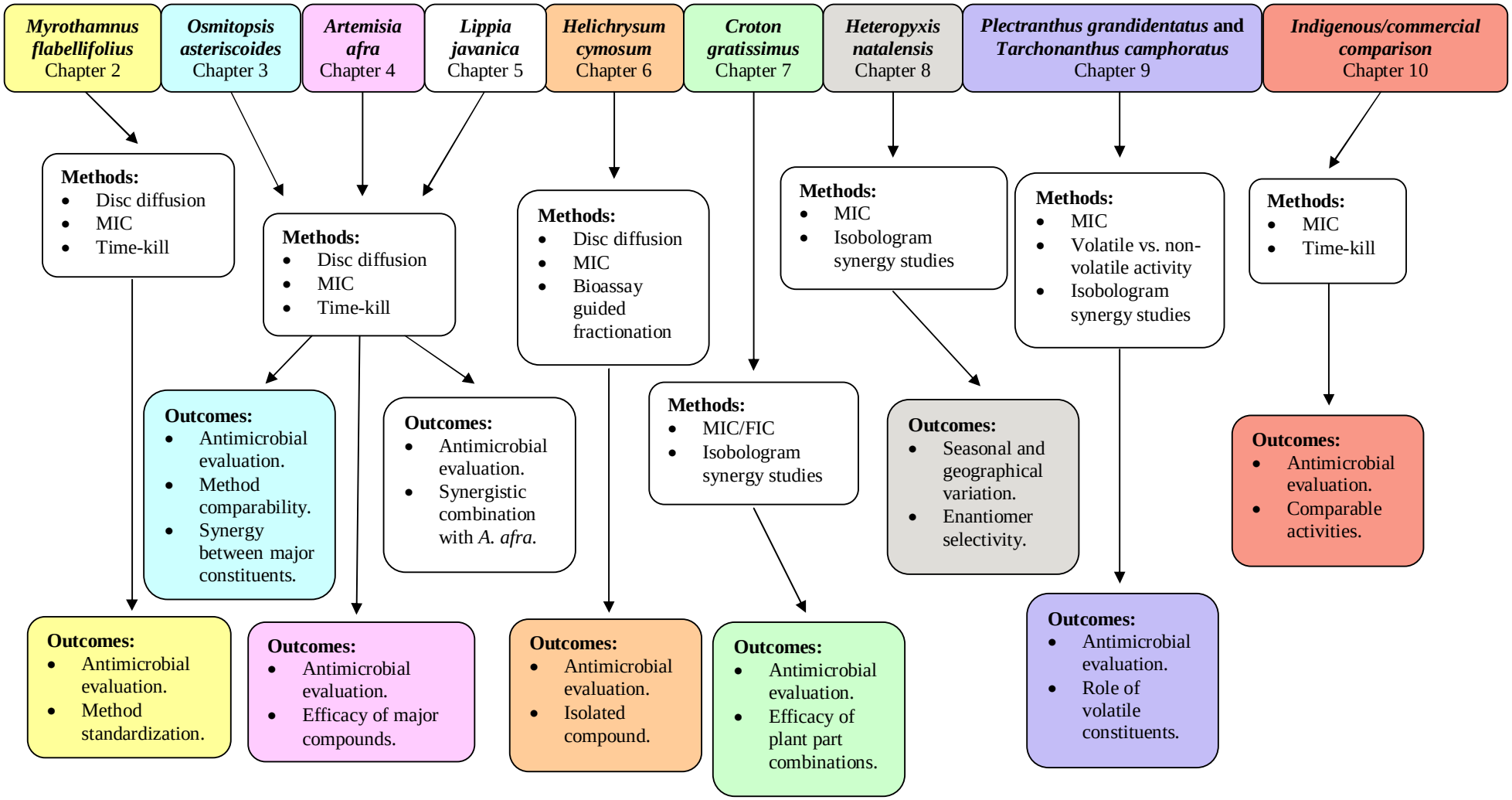


Figure 12.1 Outline of thesis: The antimicrobial activity and essential oil composition of medicinal aromatic plants used in African traditional healing.

Objective 1: To determine the antimicrobial activity of selected indigenous aromatic plants used in traditional healing.

In a review of over 500 publications detailing the antimicrobial activity of plants, no set criteria could be found that classifies an essential oil as having good, moderate or poor activity, and many researchers base the assessment on their own particular data attained. It is clear from this study that the essential oils exhibit much higher MIC values (poorer activity) than the extracts. Antimicrobial activity with MIC values 4 mg/mL or lower were considered noteworthy for these studies. Based on this criterion highest activity was attained for *M. flabellifolius* oil where seven of the nine pathogens studied exhibited MIC values of 4 mg/mL or lower. Similarly good activity was found for four of the ten pathogens studied with *H. cymosum* subsp. *cymosum* oil and four of the eight pathogens studied with *P. grandidentatus* oil.

With this study on aromatic plants, it was noted that it was not always the volatile (essential oils) constituents that were responsible for the highest antimicrobial activity, but also the non-volatile (extracts) constituents, as documented with the studies on *H. cymosum* subsp. *cymosum, C. gratissimus* var. *subgratissimus, T. camphoratus* and *P. grandidentatus*. Extracts having activities where MIC values are below 8 mg/mL (Fabry *et al*., 1998) are considered to possess some antimicrobial activity, and natural products having activities where MIC values are below 1 mg/mL are considered noteworthy (Gibbons, 2004; Rios and Recio, 2005). Antimicrobial activities for extracts with MIC values 1 mg/mL or lower were attained for all pathogens when investigated against the extract of *H. cymosum* subsp. *cymosum.* Other extracts exhibiting high activities include *C. gratissimus* var. *subgratissimus* (bark) with four of the ten pathogens having MIC values lower than 1 mg/mL and *P. grandidentatus* (dried plant material), also with four pathogens having MIC values lower than 1 mg/mL**.**

Compounds having antimicrobial activities 64-100 µg are accepted as having clinical relevance for antimicrobial activity (Gibbons, 2004; Rios and Recio, 2005). The isolated compound helihumulone from *H. cymosum* subsp. *cymosum* had MIC values below 64 µg against nine of the ten pathogens tested.

The highest antimicrobial activities obtained using MIC methodology, noted for all plants within this study are given in Table 12.1.

Table 12.1 The highest antimicrobial activities found for all plants in this study.

Plant	Component of plant	Test organism showing highest antimicrobial activity	Antimicrobially related traditional use[*1]	MIC value (mg/mL)
M. flabellifolius	Essential oil	*Cryptococcus neoformans* ATCC 90112	Colds, respiratory ailments, chest pains and coughs	2.00
O. asteriscoides	Essential oil	*Escherichia coli* ATCC 1175	Stomach complaints and intestinal disorders	4.00
		Staphylococcus epidermidis ATCC 2223	Cuts and swellings	
A. afra	Essential oil	*Serratia odorifera* ATCC 33132	Ailments associated with the gastrointestinal tract	4.00
L. javanica	Essential oil	*Cryptococcus neoformans* ATCC 90112	Coughs, colds, bronchial and respiratory ailments	4.00
		Escherichia coli ATCC 11775	Dysentery, diarrhoea and suspected food poisoning	
H. cymosum subsp. *cymosum*	Leaf extract	*Bacillus cereus* ATCC 11778	Topical infections	0.08
		Cryptococcus neoformans ATCC 90112	Respiratory ailments	
	Helihumulone	*Pseudomonas aeruginosa* ATCC 9027	Topical infections	0.02
C. gratissimus var. *subgratissimus*	1:1:1 Leaf, bark and root	*Bacillus cereus* ATCC 11778	Abdominal disorders and skin inflammation	0.30[*2]
		Staphylococcus aureus ATCC 12600	Skin inflammation	
	1:1 Bark and root	*Bacillus cereus* ATCC 11778	Abdominal disorders and skin inflammation	
H. natalensis	Essential oil (Lagalametse and Balakane samples)	*Cryptococcus neoformans* ATCC 90112	Respiratory disorders and as a decongestant	0.50
T. camphoratus	Dried leaf extract	*Candida albicans* ATCC 10231	Stomach ailments	0.30
P. grandidentatus	Dried leaf extract	*Enterococcus faecalis* ATCC 29212	Respiratory complaints	0.06

[*1] Adapted from reference literature within relevant Chapters.
[*2] *Bacillus subtilis* is excluded from data set due to the organism's rare pathogenic status.

Objective 2: To record the essential oil composition of some of the most widely used South African medicinal aromatic plants.

The major constituents for each plant essential oil are summarized in Table 12.2. The most predominant constituent present in five of the nine plants studied was 1,8-cineole. This monoterpene compound has previously attained pharmacological status as an inhalant and expectorant with application for respiratory complaints (Windholz, 1976; Silvestre *et al*., 1999). Thus, it is not surprising that it is associated with the aromatic plants studied here. The antimicrobial property of 1,8-cineole has also been widely investigated (Table 12.3) with varied reports attained by different authors. Variation was noted between strains having the same reference number as well as strains having different reference numbers. Such variations emphasise the need to standardize reference cultures, as well as corroborate findings between different laboratories. Mostly the authors are in general agreement that some antimicrobial efficacy may be attributed to 1,8 cineole. One must not forget however, that synergistic interactions between constituents may occur, such as that noted in the *O. asteriscoides* study where 1,8-cineole in combination with (-)-camphor act synergistically to enhance the antimicrobial activity.

Objective 3: To investigate the plant composition with antimicrobial activity relating to structure activity relationships and if possible identify the antimicrobial factor responsible.

Results observed from the microbiological activity for the various oils have shown that the correlation between chemical structure and biological activity are integrated and the essential oil chemistry has provided insights into the antimicrobial activity. Studies on the structure activity related antimicrobial activity was demonstrated with the interaction of the major oil constituents of *O. asteriscoides,* where the combination of camphor and 1,8-cineole were shown to enhance antimicrobial efficacy. Investigation of the four major compounds most abundant in the essential oil of *A. afra* (artemisia ketone, 1,8-cineole, α and β-thujone) indicated minimal antimicrobial activity against *K. pneumoniae* after 24 hours, when investigated independently and in various combinations. It is probable that the mono- and sesquiterpenes could exert antimicrobial properties by working in a synergistic manner or that minor compounds greatly contribute to the antimicrobial activity. Studies on the essential oil, extract and isolated biological compound helihumulone from *H. cymosum* subsp. *cymosum* demonstrated higher activities for the extract.

Helihumulone, not found in the volatile essential oil, could possibly indicate why the essential oil showed lower antimicrobial activities than the crude extract. Enantiomer studies, as undertaken with *H. natalensis* indicated how the variation of results obtained between the (+), (-) and racemic forms of limonene when combined with 1,8-cineole as well as their combination in different ratios clearly impact on the bio-activity. The geographical investigation of the *H. natalensis* oils revealed that the anomalous chemotype from Lagalametse having (*Z*)-3-hexenyl nonanoate as a major constituent could possibly account for the increased antimicrobial activity found in this sample. In a recent publication by Holley and Patel (2005), mention was made of the lack of information available on essential oil constituent interactions. The work presented here sheds some light on these possible interactions.

Table 12.2 Summary of the major oil constituents obtained from all plants in this study.

Plant essential oil	Major components	Component %	% within total essential oil
M. flabellifolius	*trans*-pinocarveol	19.6	30.7
	pinocarvone	11.1	
O. asteriscoides	1,8-cineole	59.9	72.3
	(-)-camphor	12.4	
A. afra	1,8-cineole	17.8	59.2
	artemisia ketone	10.1	
	α-thujone	18.8	
	β-thujone	12.5	
L. javanica	linalool	65.2	78.2
	(*Z*)-β-ocimene	13.0	
H. cymosum subsp. *cymosum*	1,8-cineole	20.4	43.6
	β-caryophyllene	10.8	
	α-pinene	12.4	
C. gratissimus var. *subgratissimus*	1,8-cineole	8.3	37.6
	germacrene D	8.6	
	α-phellandrene	20.7	
H. natalensis	limonene	15.1-25.4	Variable*
	linalool (Nelspruit and Lagalametse samples)	11.4-16.7	
	(*Z*)-3-hexenyl nonanoate (Lagalametse sample)	16.0	
	ß-pinene (Balakane sample)	25.2	
T. camphoratus	1,8-cineole	9.3	22.7
	β-caryophyllene	13.4	
P. grandidentatus	T-cadinol	10.3	25.5
	camphor	15.2	

*Composition % within major constituents displayed seasonal and geographical variation.

Table 12.3 The antimicrobial properties of 1,8-cineole as determined by corresponding authors.

Publication	Test micro-organism with corresponding reference strain number						
	S. aureus	***S. epidermidis***	***B. cereus***	***E. coli***	***P. aeruginosa***	***K. pneumoniae***	***C. albicans***
Carson and Riley (1995)	0.5 (NCTC 6571)	ND	ND	0.25 (NCTC 8359)	ND	ND	1.0 (ATCC 10231)
Griffin *et al.* (1999)	18.1 (NCTC 8325)	ND	ND	9.1 (AG100)	18.1 (NCTC 6749)	ND	9.1 (KEMH 5)
Tzakou *et al*. (2001)	9.5 (ATCC 25923)	9.5 (ATCC 12228)	ND	2.0 (ATCC 25922)	2.8 (ATCC 227853)	3.0 (ATCC 13883)	0.3 (not given)
Magiatis *et al*. (2002)	9.5 (ATCC 25923)	9.5 (ATCC 12228	ND	2.4 (ATCC 25922)	2.8 (ATCC 227853)	3.2 (ATCC 13883)	0.3 (ATCC 10231)
Setzer *et al*. (2004)	1.3 (ATCC 29213)	ND	0.3 (ATCC 14579)	0.6 (ATCC 25922)	0.6 (ATCC 27853)	ND	0.6 (ATCC 10231)
Cha *et al.* (2005)	12.8 (ATCC 21293)	0.8 (ATCC 12228)	ND	3.2 (ATCC 25922)	ND	ND	ND
van Vuuren (Chapter 8)	8.0 (ATCC 12600)	ND	2.0 (ATCC 11778)	8.0 (ATCC 11775)	4.0 (ATCC 9027)	8.0 (ATCC 13883)	ND

ND = not determined.

Objective 4: To study specific pharmacological interactions.

The topic of synergy has been highlighted recently by many authors. In the synergy review by Williamson (2001), the need for further interactive investigations was emphasized. Rios and Recio (2005) recommended that a high priority should be given to further studies involving plant combinations and Gilani and Rahman (2006) amplified that there is a scarcity of ethnopharmacognosists performing a much needed study into medicinal plant combinations. Holley and Patel (2005) stated that very little information was available on constituent interaction. The emphasis on synergistic studies was also highlighted by Prof. H Wagner (Editor: Phytomedicine) at the Annual Conference of the Society for Medicinal Plant Research, Florence (21-25 August, 2005) when he stated "Progress in the field of synergy research could give phytotherapy new legitimacy and the possibility to treat diseases which up to now were reserved for chemotherapy only".

This study thus identified possible interaction between various constituents of the essential oils as was noted with the interaction of the major oil constituents of *O. asteriscoides* where camphor and 1,8-cineole, in combination act synergistically to enhance activity. Conversely, the major volatile constituents of *A. afra* in various combinations had no significant role on the antimicrobial activity of the plant. The interaction between different plant species was studied using time-kill methodology, where the synergistic interaction was demonstrated for the combined use of *L. javanica* with *A. afra* for the treatment of respiratory infections associated with *K. pneumoniae*. Results show that in combination, efficacy is enhanced, which supports with the traditional use of the plant. Antimicrobial studies on *C. gratissimus* var. *subgratissimus* show synergistic, additive, antagonistic or non-interactive action between plant parts, depending on the specific ratio in which the plant parts are combined. Higher synergistic sensitivities were noted for the root / leaf combinations. Other interactive studies have shown that the volatile fractions within *T. camphoratus* and *P. grandidentatus* serve to enhance overall activity of the plant.

Objective 5: To compare the antimicrobial activity (potency) of indigenous aromatic plants to commercially available essential oils with claimed antimicrobial activity.

Chapter 10 deals exclusively with this objective. By means of comparatively determining the MIC of popular commercial oils (*Lavendula angustifolia*, *Thymus vulgaris*, *Melaleuca*

alternifolia, Mentha piperita and *Rosmarinus officinalis*) against the essential oils of five indigenous plants (*M. flabellifolius, O. asteriscoides, H. natalensis, A. afra* and *L. javanica*), efficacy was determined against eight bacterial reference strains and two yeast reference strains. The laboratory conditions and inoculum were standardized to ensure all ten essential oils (commercial and indigenous) were evaluated under identical conditions. Results indicated that South African indigenous essential oils compare favourably with the commercial oils studied herein. Of all oils studied, *M. flabellifolius* showed the most rapid cidal effect against all three pathogens tested with the time-kill method.

Objective 6: To compare and validate results obtained from various test methodologies (e.g. disc diffusion, MIC and time-kill studies).

Many variables are involved in plant-based studies and microbiological assays are only as reliable and sensitive as the test system employed. It was therefore necessary to incorporate more than one microbiological method to validate the results attained. The methods used for each study is summarised in Figure 12.1. Detailed methodology development and critical validation assessment was reviewed in Chapter 11.

Objective 7: To provide a scientific rationale for the traditional use of various medicinal aromatic plants to treat infectious diseases.

Incorporated in Table 12.1 is a summary of the traditional use of the plants studied with reference to the test organisms having the most significant activity. Other significant findings were noted for plant combinations, as was the case for the combination of *L. javanica* and *A. afra,* where the time-kill study indicated increased antimicrobial efficacy for the respiratory pathogen *K. pneumoniae.* These findings support the traditional use of these plants when used together in traditional therapeutic practices.

The benefit of combining roots and leaves of *C. gratissimus* var. *subgratissimus* as used traditionally was also apparent in the isobologram studies, where extracts showing the strongest synergistic profile were noted with studies on *B. cereus, C. albicans* and *C. neoformans,* correlating with diseases that the plant is traditionally used to treat.

12.3 Future Trends

Antimicrobial studies on plants have evolved from multidisciplinary investigations where researchers have used the antimicrobial data to complement the phytochemical study, to focused studies pertaining to specific information about the plants' antimicrobial activity. This reflects the importance of such studies and conti nued investigations have emerged as a result of many of these positive preliminary screening procedures. A number of recommended strategies for future
antimicrobiological analysis in pharmacognosy should thus be considered.

Toxicity: Although it is considered that traditional plant medicines, having been used for centuries, are generally safe, it can not be taken for granted that these treatments do not have any potentially toxic, mutagenic and / or carcinogenic properties and thus it has been recommended that pharmacological studies should always be accompanied by toxicology screening (Taylor *et al*., 2001; Fennel *et al*., 2004; Cos *et al*., 2006). The toxicology of the oils studied have all, with the exception of *P. grandidentatus* (still in progress), been assessed by Dr. Robyn van Zyl at the Pharmacology Division of the Department of Pharmacy and Pharmacology, University of Witwatersrand. Toxicology data (Table 12.4) is arranged in ascending order from the least toxic (*O. asteriscoides*) to the most toxic (*C. gratissimus* var. *subgratissimus*) plant species. Toxicity comparisons were made with quinine, considered as an acceptable control, presenting low toxicity.

It comes as no surprise that *C. gratissimus* var. *subgratissimus* demonstrates the highest toxicity profile as this species is known for its toxicity (Watt and Breyer-Brandwijk, 1962; Hutchings *et al*., 1996). The presence of copious amounts α-phellandrene (20.7%) in the oil may also contribute towards the overall toxicity, as this compound is known for its skin irritancy effect and ability to cause vomiting and diarrhoea if ingested (Windholz, 1976). A further recommendation to investigate the toxicity profiles for the phenolic extracts of *C. gratissimus* var. *subgratissimus* (bark, leaves and roots), *T. camphoratus* and *P. grandidentatus* is advised.

Table 12.4 The toxicity profiles for the eight essential oils studied (van Zyl, publication in preparation).

Essential oils	Mean toxicity profile IC_{50} (%)
Osmitopsis asteriscoides	0.00908 ± 0.00140
Lippia javanica	0.00730 ± 0.00065
Artemisia afra	0.00522 ± 0.00088
Heteropyxis natalensis	0.00246 ± 0.00032
Myrothamnus flabellifolius	0.00226 ± 0.00010
Tarchonanthus camphoratus	0.00214 ± 0.00112
Helichrysum cymosum subsp. *cymosum* *	0.00175 ± 0.00310
Croton gratissimus var. *subgratissimus*	0.00157 ± 0.00016
Quinine control	0.01360 ± 0.00041

*(van Vuuren *et al*., 2006)

Mode of action studies: While investigations presented here demonstrate the microbial inhibition on a selection of indigenous medicinal plant essential oils, the mechanism of action requires further insight. Mode of action studies are complex and destruction of microbial cells could occur on many different levels. There is no doubt that essential oils compromise membrane integrity, as noted in studies on *O. asteriscoides* (Chapter 3) and with studies by Cox *et al*. (2000), Griffin *et al*. (2001), Lambert *et al*. (2001) and Fitzgerald *et al.* (2004), however, there are a number of other mechanisms that need to be elucidated. Leakage of potassium ions and nucleic acids from bacterial cells (Hada *et al*., 2003; Inoue *et al*., 2004), impairment of a variety of enzyme systems, electrochemical potential, measurement of microbial respiration (Cox *et al*., 2000), ATPase activity (Burt, 2004), cellular metabolic processes (Holley and Patel, 2005), changes in composition of fatty acid and intracellular absorption of material (personal communication Dr. Panos Skandamis) and even the role of constituents as penetration enhancers require further investigation. One also needs to take into consideration that given the number of essential oil constituents within one plant, there may be constituents independently exhibiting different modes of action that when combined, act synergistically.

Dosage schedules and administration for indigenous medicines: Research design often overlooks these factors, which may be in part due to the lack of information provided to researchers on the details of the traditional use. But moreover, laboratory techniques often exclude aqueous extracts, which is the most widely used traditional administration, in favour of working with apolar extracts in order to maximize activities. Technically, aqueous extracts are more difficult to work with and especially with microbiological work, contaminate rapidly, however, these studies are important as bioactive compounds may be overlooked. In addition, positive results from laboratory tests may indicate activities greater than that found in the traditional preparative methods, as indicated in a study by Jäger (2003). With the rapid rate of plant species extinction, there is an urgent need to document the medicinal potential of plants in an effort to preserve the knowledge and natural resources. It is a future challenge to translate the applied knowledge gained from intricate assays and make it meaningful to the ethnic people.

Future research design should also look at regular or consistent administration of antimicrobials to determine if better efficacy would be achieved with sub chronic administrations in comparison with single acute administrations (Etkin, 2001; Etkin and Elisabetsky, 2005). As in scheduled antimicrobial therapy, consistent dosages are required to produce a cidal effect. Such studies could be carried out with plant test extracts or oils in extended time-kill methods, where the viability can be monitored over time with regular dosages.

Pharmacological interactions: The identification of a single active chemical entity that is responsible for the antimicrobial activity of a plant is becoming more and more improbable. Research is now focussing on the investigation of a combination of compounds to achieve a greater efficacy. To ensure future success in natural product research we must incorporate interactive phytochemical studies with existing practices in the hope that developments involving combinations may be used as a foundation and driving force in the search for novel chemotherapeutic agents.

Combinations with conventional antimicrobials: Studies supporting the possible use of medicinal plants together with known antibiotics may serve to increase potency and reduce the dosage of potent drugs. In so doing, the toxicity levels may be reduced. With natural product research gaining credibility and the escalated use of herbal remedies by the public sector, less burden and reliance may be placed on conventional antimicrobial drugs. In so doing we can

preserve these important allopathic antimicrobials and delay the emergence of resistance. In a recent publication by Rios and Recio (2005), this proposal is given high priority.

Metabolomics: The metabolomic study of plants is a rapidly growing field, where the metabolites of plant species are quantitatively and qualitatively analyzed in order to obtain more information on the overall mechanism of action. Information regarding metabolites can be correlated with biological activities to determine if a specific fingerprint exists. Such studies encompass all aspects of plant metabolites and are a major challenge as this moves away from the single targeted bioactivity approach of drug development (Verpoorte *et al.*, 2005).

Pathogen specific studies: More attention needs to be directed towards the ethnobotanical use of the plant and the rational antimicrobial screening that follows. Plants used for skin afflictions should be tested against micro-organisms such as *Epidermophyton floccosum, Microsporum canis* and *Trichophyton mentagrophytes*, in addition to the more commonly tested organisms such as *S. aureus* and *S. epidermidis*. Similarly, plants used for oral complaints should be tested against *Streptococcus mutans, S. sobrinus* and *Porphyromonas gingivalis*. With studies on plants used for the treatment of sexually transmitted diseases, choice of bacterial and fungal test strains are limited. While *Neisseria gonorrhoeae* remains the most commonly studied organism, other organisms responsible for sexually transmitted infections (STI) include *Treponema pallidum* (Syphilis) and *Haemophilus ducreyi* (Chancroid). Studies on *T. pallidum* prove difficult as the micro-organism cannot be cultured in common bacteriological growth media. Studies on *H. ducreyi* are no longer feasible as outbreaks no longer occur (personal communication Prof. Lewis at the National Centre for Communicable Diseases), however, in spite of the difficulties associated with culturing of sexually transmitted organisms, there is a need to investigate the use of medicinal plants for the treatment of STI's, even if studies are only restricted to *N. gonorrhoeae*.

Whilst it is understandable that all these organisms mentioned here are fastidious and require more intensive cultural techniques, it is a necessary progression for further antimicrobial pharmacognosy studies. We can no longer assume that broad spectrum activity is adequate for plants used for specific afflictions.

***In vivo* and whole system studies:** Even though a number of successful studies have identified specific plant species as having antimicrobial activity in an *in vitro* model, it is necessary to

subject these plants to animal and human studies to determine their efficacy in whole organism systems. Traditional medicines are esteemed not only for their therapeutic value but also from a holistic administration approach, in which the plant is given to treat the patient on various levels. Thus, when studying traditional medicines one must not forget that there may be other physiological effects on the body that act beyond the symptomatic treatment of the disease.

In addition, studies on the efficacy of plants against resident beneficial bacteria such as *Lactobacillus acidophilus* and *Bifidobacterium bifidum* could yield information that may make them more appropriate than the presently administered allopathic antimicrobials, which inevitably destroy the beneficial commensal organisms together with the invading pathogens. In line with this, studies on biofilms should be initiated as these mixed populations play an active role in the microbiological ecology of the human body. Ethnopharmacological studies have shown that many plant essential oils are broad spectrum in their activity, unlike conventional chemotherapeutic agents which often target specific bacterial modes of action. This multi-faceted approach may be effective in treating polymicrobial infections and may also prove to be effective in inhibiting undesirable biofilm formation.

Formulation studies: The next logical step in the investigation of the antimicrobial efficacy of plant extracts and essential oils would be to establish suitable formulations that retain the efficacy demonstrated in the *in vitro* screening procedures. Formulations such as tinctures, teas, ointments, capsulation or tableting incorporating plant material should be investigated to determine feasibility and bioavailability.

12.4 Conclusion

The antimicrobial study of some of the most significant indigenous aromatic plants presented here focuses not only on antimicrobial screening but also on addressing many of the concerns and recommendations mentioned in literature. By addressing the complexity of standardizing various methods, as recomme nded by Burt (2004) as well as Rios and Recio (2005), many of the variables encountered when performing antimicrobial assays were examined. Furthermore the interaction between constituents, plant parts, as well as different species, emphasises that the reductionist approach used in examining bio-activity may often be short-sighted (Rios and Recio, 2005; Holley and Patel, 2005; Verpoorte *et al.*, 2005; Cos *et al*., 2006).

It is interesting to note that amongst all the modern technologically advanced medical treatments available today, it is still the simplest bacterium that may result in humankind's demise. One can only hope that with continued research we can devise mechanisms that may delay or prevent this from happening. We need to plan for future therapeutic treatments that will withstand the growing population needs and emergence of "superbugs" such as MRSA and VRE. In support of this, the study of the antimicrobial activities of medicinal plants has clearly become a progressive trend. It has been predicted (Mulholland, 2005) that multidisciplinary studies within the ethnopharmacology sector will escalate within the next decade. With advances in laboratory techniques, renewed interest in the field and the scientific validation of the traditional use, the possibility now exists to bring traditional medicine to such a level of recognition that it becomes accepted alternate regimen to western healthcare systems. The anticipated future of antimicrobial phytotherapy should not attempt to replace conventional antimicrobials but rather focus on the integration into conventional medicinal practices where the general practitioner may recommend either phytomedicine or allopathic medicine or even both, depending on the diagnosis (Tyler, 1999).

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Appendix A

Raw MIC data (mg/mL) for isobolograms

Table A1 Raw MIC data (mg/mL) for isobolograms (Chapter 7) where C. *gratissimus* roots and leaves were combined.

Ratio	Combined MIC (roots and leaves) in combination (mg/mL)									
	B. cereus	***S. aureus***	***S. epidermidis***	***B. subtilis***	***E. faecalis***	***K. pneumoniae***	***P. aeruginosa***	***E. coli***	***C. albicans***	***C. neoformans***
100:0	0.8	2.0	2.0	3.0	4.0	2.0	2.0	4.0	8.0	0.5
90:10	1.0	4.0	2.0	8.0	2.0	4.0	4.0	16.0	8.0	0.5
80:20	1.0	2.0	2.0	4.0	2.0	2.0	2.0	8.0	4.0	0.5
70:30	1.0	2.0	2.0	4.0	2.0	2.0	2.0	4.0	3.0	0.6
60:40	1.5	2.0	2.0	4.0	4.0	2.0	2.0	4.0	4.0	0.5
50:50	0.5	2.0	2.0	4.0	1.4	2.0	2.0	4.0	3.0	0.5
40:60	0.5	2.0	2.0	2.0	4.0	2.0	2.0	4.0	3.2	0.3
30:70	0.5	2.0	2.0	2.0	4.0	2.0	2.0	4.0	3.0	0.5
20:80	1.0	2.0	2.0	2.0	4.0	2.0	2.0	4.0	3.0	0.5
10:90	2.0	2.0	2.0	2.0	4.0	2.0	2.0	4.0	8.0	1.0
0:100	2.0	2.0	2.0	3.0	4.0	2.0	2.0	4.0	3.0	1.0

Table A2 Raw MIC data (mg/mL) for isobolograms (Chapter 8) where (+), (-) and the racemic form of limonene was combined with 1,8-cineol.

Ratio	Combined MIC of limonene in combination with 1,8-cineol (mg/mL)								
	S. aureus			*P. aeruginosa*			*C. neoformans*		
	(+)-limonene	(-)-limonene	(+/-)-limonene	(+)-limonene	(-)-limonene	(+/-)-limonene	(+)-limonene	(-)-limonene	(+/-)-limonene
100:0	16.0	4.0	4.0	4.0	8.0	4.0	2.0	2.0	2.0
90:10	16.0	16.0	8.0	6.0	8.0	8.0	4.0	2.0	4.0
80:20	16.0	9.6	6.0	6.0	8.0	8.0	4.0	2.0	2.0
70:30	16.0	10.4	6.0	6.0	8.0	4.0	2.0	2.0	2.0
60:40	7.0	17.6	6.0	4.0	8.0	4.0	2.0	2.0	2.0
50:50	8.0	8.0	6.0	4.0	8.0	4.0	2.0	2.0	2.0
40:60	8.0	8.0	6.0	4.0	8.0	4.0	2.0	2.0	2.0
30:70	8.0	8.0	8.0	4.0	8.0	4.0	2.0	2.0	2.0
20:80	8.0	8.0	8.0	4.0	8.0	4.0	2.0	2.0	2.0
10:90	8.0	8.0	8.0	4.0	8.0	4.0	2.0	2.0	3.0
0:100	8.0	8.0	8.0	4.0	8.0	6.0	2.0	1.5	2.0

Table A3 Raw MIC data (mg/mL) for isobolograms (Chapter 9) where the volatile and non-volatile constituents for *T. camphoratus* were combined.

Ratio	Combined MIC (volatile and non-volatile) in combination (mg/mL) for *T. camphoratus*							
	B. cereus	***S. aureus***	***E. faecalis***	***K. pneumoniae***	***P. aeruginosa***	***E. coli***	***C. albicans***	***C. neoformans***
100:0	3.0	4.0	8.0	8.0	4.0	8.0	6.0	3.0
90:10	1.0	2.0	4.0	2.0	8.0	8.0	2.0	1.5
80:20	1.0	1.5	4.0	2.0	3.0	4.0	2.0	1.5
70:30	1.0	1.5	4.0	4.0	3.0	4.0	2.0	2.0
60:40	0.7	1.5	4.0	4.0	3.0	4.0	2.0	2.0
50:50	0.5	1.5	4.0	4.0	3.0	4.0	2.0	2.0
40:60	0.5	1.0	4.0	4.0	4.0	8.0	2.0	2.0
30:70	0.5	1.0	4.0	4.0	4.0	8.0	2.0	1.0
20:80	0.5	1.0	4.0	4.0	4.0	8.0	2.0	1.0
10:90	0.5	1.0	4.0	4.0	4.0	8.0	3.0	1.0
0:100	2.0	2.0	4.0	8.0	4.0	4.0	4.0	3.0

Table A4 Raw MIC data (mg/mL) for isobolograms (Chapter 9) where the volatile and non-volatile constituents for *P. grandidentatus* were combined.

Ratio	Combined MIC (volatile and non-volatile) in combination (mg/mL) for *P. grandidentatus*		
	S. aureus	***E. faecalis***	***K. pneumoniae***
100:0	2.0	8.0	4.0
90:10	0.3	1.0	2.0
80:20	0.5	1.0	2.0
70:30	0.5	1.0	1.0
60:40	0.5	1.0	1.0
50:50	0.5	2.0	1.0
40:60	0.2	4.0	1.0
30:70	1.0	1.0	1.0
20:80	0.5	3.0	1.0
10:90	1.0	4.0	1.0
0:100	0.4	32.0	8.0

Appendix B

Publications arising from this study