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2012-01-17
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Antimicrobial interactions of *Artemisia afra* used in African traditional medicine

Sajida Suliman



A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfillment of the requirements for the Degree of Master of Pharmacy

Johannesburg, 2011

Declaration

I, Sajida Suliman, declare that this dissertation is my own work. It is being submitted in fulfillment for the degree of Master of Pharmacy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

Sajida Suliman

Date

Abstract

Many therapies prescribed by traditional healers in Southern Africa include plant combinations to treat infectious diseases. *Artemisia afra* is one of the most commonly used traditional medicines in African traditional medicine and most often given in combination with other plants. This plant's popularity coupled with its wide range of uses in combination serves as the rationale for the bases of this study. In this study, combinations of *A. afra* (essential oils and plant extracts), which are commonly used for the treatment of respiratory diseases were studied from an antimicrobial perspective in order to determine if a scientific basis exists for their combined use. The plants used often in double or triple combination with *A. afra* in the treatment of respiratory tract infections are *Lippia javanica*, *Osmitopsis asteriscoides*, *Agathosma betulina*, *Eucalyptus globulus*, *Allium sativum*, *Leonotis randii*, *Tetradenia riparia* and *Zanthoxylum capense*.

Essential oils from plant samples were analysed using gas chromatography coupled to mass spectroscopy (GC-MS). Compounds found in highest concentrations were camphor (41.0%) in *A. afra*, linalool (70.7%) in *L. javanica*, 1,8-cineole (59.0%) in *O. asteriscoides*, isomenthone (31.4%) in *A. betulina*, 1,8-cineole (63.0%) in *E. globulus* and β-caryophyllene (32.4%) in *T. riparia*.

Dichloromethane: methanol extracts and aqueous extracts were prepared for each plant using the dried ground plant material collected. The antimicrobial activities of each sample as well as each combination (including essential oils) were tested using the minimum inhibitory concentrations (MIC) assay against a panel of respiratory tract organisms. The highest sensitivities observed for the essential oils were that of *E. globulus* against *Cryptococcus neoformans* with a MIC value of 0.6 mg/ml. The dichloromethane: methanol extracts showed the most activity with *E. globulus* against *Moraxella catarrhalis* (MIC value of 0.01 mg/ml). The aqueous extracts showed the best activity with *Z. capense* against *Streptococus agalactiae* with a MIC value of 0.4 mg/ml.

The 1:1 fractional inhibitory concentration (∑FIC) values of the combinations of *A. afra* with *L. javanica*, *A. afra* with *O. asteriscoides*, *A. afra* with *A. betulina*, *A. afra* with *E. globulus* and *A. afra* with *Z. capense* were calculated from the MIC data. Synergy, additivity,

indifference and antagonistic interactions within the combinations were then interpreted. The most significant interactions of the double combinations with synergistic ∑FIC values of 0.2 were the combination of the dichloromethane: methanol extracts of *A. afra* with *O. asteriscoides* against *Streptococcus pyogenes* and the combination of the aqueous extracts of *A. afra* with *E. globulus* against *Streptococcus pneumoniae*. Significant antagonism was noted with the combination of the dichloromethane: methanol extracts of *A. afra* with *E. globulus* against *Enterococcus faecalis*.

The ∑FIC results of the combinations of *A. afra* with *L. javanica*, *O. asteriscoides*, *A. betulina*, *E. globulus* or with *Z. capense* were used to calculate ratios and plotted on to an isobologram. The isobolograms were interpreted with regard to any synergy, antagonism, or additive interactions present in the combination. Isobolograms revealed the most significant activity with the combination of the aqueous extracts of *A. afra* with *E. globulus* against *C. neoformans* with all the ratios tested being synergistic. The most prominent antagonism (five ratios) noted was in the combination (dichloromethane: methanol extracts) of *A. afra* with *E. globulus* against *M. catarrhalis*.

The triple combinations analyzed for their antimicrobial activity were the combinations of *A. afra* with *O. asteriscoides* and *E. globulus*, *A. afra* with *L. randii* and *E. globulus*, *A. afra* with *A. sativum* and *Z. capense* and the combination of *A. afra* with *T. riparia* and salt. The most significant synergistic activity was noted for the combination of the essential oils *A. afra* with *T. riparia* and salt against *Mycobacterium smegmatis* with a ∑FIC value of 0.2. The combination of *A. afra* with *O. asteriscoides* and *E. globulus* of (dichloromethane: methanol extracts) displayed the most antagonistic activity against *M. catarrhalis*.

When analysing the combinations that include *A. afra*, it was noted that adjuncts are an important combination ingredient in the traditional method of preparation. These were also tested for their activity. The combinations that include adjuncts i.e. honey, salt, vinegar, brandy and milk showed mainly indifferent interactions. This indifference noted supports the use of these adjuncts by traditional healers as it serves to verify that these adjuncts are at least not hindering the activity of the plant itself, which is a positive direction for future investigations.

Traditional medicine, with regard to *A. afra*, as prescribed by traditional healers, has commonly employed the use of combinations of more than one plant to treat respiratory conditions. When the antimicrobial activities in combination were examined from a scientific viewpoint, there is evidence of some bases for their traditional use. The results obtained from the testing of the essential oils validate its traditional use as an inhalant. The dichloromethane: methanol extracts showed results varying from synergy to antagonism while the aqueous extracts showed good antimicrobial activity. It is recommended that future studies should be conducted into these interactions to determine the benefits of these combinations for possible use in the commercial and primary health-care sectors.

Dedication

To my parents, Noormohamed and Farida, who have always supported me in my endeavor to achieve success.

To my sister, Suraiya, for your unwavering faith and confidence in my abilities.

To my nieces Sameeha and Asma, anything is possible.

"The courage of life is often a less dramatic spectacle than the courage of a final moment, but it is no less than a magnificent mixture of triumph and tragedy. People do what they must – in spite of personal consequences, in spite of obstacles and dangers and pressures – and that is the basis of all human morality"

John F. Kennedy

Acknowledgements

In The Name of Allah (God), the Most Beneficent, the Most Merciful

- Before all, I must first give thanks to my creator and sustainer for guiding me along the path of knowledge and giving me the inner strength and determination to complete this piece of work. This dissertation has shown me that faith is as important a tool in life as skill and determination.

- To my supervisor, Associate Professor Sandy van Vuuren, I thank you from the very bottom of my heart. What I have learnt from you these past few years has been more than simply microbiology and pharmacognosy, but I have learnt the value of hard work, dedication and perseverance. All that I have learnt from you as a Professor as well as a mentor is invaluable.

- To my co supervisor, Professor Alvaro Viljoen, I thank you for your wisdom and boundless knowledge in the field of botany, which proved to be of great value to this research project. My dissertation would not look complete without your photographs so I thank you for providing some of the photographic material of the plants used in my study.

- I would also like to thank all the staff members at the Department of Pharmacy and Pharmacology of the University of the Witwatersrand for all their kind assistance with various tasks.

- Many thanks also go out to the University of the Witwatersrand Health Sciences Library and its willing staff members for their assistance in literature acquisition.

- Thank you to Joanet Maree at the Tshwane University of Technology for all the help with the TLC analysis as well as Dr Guy Kamatou for the assistance with the GC-MS.

- The Walter Sisulu Botanical gardens and its staff, in particular Mr Andrew Hankey, were always helpful and hospitable during my visits to collect plant materials. Their understanding and patience is much appreciated.

- Thank you to the National Research Foundation for their generous funding of my research.

- I would also like to thank the University of the Witwatersrand for funding provided.

- To all my friends and colleagues who have been there with me every step on this journey, I thank you for your support and understanding.

- To my sister, Suraiya thank you for your words of wisdom, unwavering support and constant words of encouragement.

- To my brother, Mohamed Ameen, for standing by my side through those long nights and early mornings, thank you.

- I am grateful to my brother Ahmed, and my sister-in law Basheera for giving me encouragement throughout my journey and letting me not waiver in my determination to achieve success.

- To my nieces Sameeha and Asma, you are the coolness of my eyes, thank you for helping me to see the beauty of life and for giving me hope.

- Last but certainly not least, I would like to extend my appreciation and warm gratitude to my parents for their continuous support, financially and for their guidance and wisdom. Mere words are not enough to describe how much I owe to them for everything in my life and I sincerely hope that they understand the gratitude I feel towards them.

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Equation 2.1

Volume of Acetone required (μl)

$$= \frac{\text{Mass of Volume of Essential oil/extract (mg)}}{\text{128mg/ml (essential oils)or 64mg/ml (extracts)}} \text{ x } 1000$$

54

Equation 2.2

$$FIC_a = \frac{\text{MIC of (a) in the combination with (b)}}{\text{MIC (a)independently}}$$

58

Equation 2.3

$$FIC_b = \frac{\text{MIC of (b) in the combination with (a)}}{\text{MIC (b)independently}}$$

58

Equation 2.4

$$FIC_{index} = FIC_a + FIC_b$$

58

Equation 2.5

$$X = \frac{\text{MIC value of combination of essential oil/extract}}{\text{MIC value of essential oil/extract}}$$

59

Equation 2.6

$$Y = \frac{\text{MIC value of combination of essential oil/extract}}{\text{MIC value of essential oil/extract}}$$

59

Abbreviations

AIDS- Acquired Immune Deficiency Syndrome

ATCC- American Type Culture Collection

CFU- Colony Forming Units

CLSI- Clinical and Laboratory Standards Institute

DC- Combined volatile and non- volatile constituents that have been prepared with fresh plant material

DMSO- Dimethyl Sulfoxide

DNA- Deoxyribonucleic acid

eV- Electron Volt

FIC- Fractional Inhibitory Concentration

g- Gram

GC- MS- Gas Chromatography Coupled with Mass Spectroscopy

HPLC- High Performance Liquid Chromatography

HPTLC- High Performance Thin Layer Chromatography

hrs-Hours

INT- *p*-Iodonitrotetrazolium Violet

min- Minutes

MIC- Minimum Inhibitory Concentration

mg- Milligram

ml- Millilitres

m/z- Mass to Charge ratio

MRSA- Methicillin Resistant *Staphylococcus aureus*

ND- Not Determined

nm- Nano-metres

NHLS- National Health Laboratory Services

psi- Pounds per Square inch

R_f- Distance travelled by spot from baseline with respect to solvent front

RRI- Relative Retention Index

SAMF- South African Medicines Formulary

SANBI- South African National Biodiversity Institute

TIC- Total ion Chromatogram

TLC- Thin Layer Chromatography

Tr- Trace

UV- Ultra Violet

WHO- World Health Organisation

µl- Micro-litre

µm- Micro-metre

°C- Degrees Celsius

Glossary

Extracts: liquid, powdered or viscous crude mixtures of chemical compounds, extracted from plant material using water or organic solvents e.g. alcohol (ethanol). (van Wyk and Wink, 2004). Thus, the extract contains only the soluble portion of the plant material and the non-soluble (fibrous) residues are discarded. (van Wyk and Wink, 2004).

Infusion: (*infusum*) this refers to a preparation that is made by adding boiling water to the required amount of drug. This preparation is then allowed to steep for five to ten minutes before it is strained. An infusion is sometimes referred to as a "tea". (van Wyk and Wink, 2004)

Decoction: (*decoctum*) this refers to a preparation that is made by adding cold water to the required amount of drug. It is then heated to boiling and allowed to simmer for five to ten minutes, thereafter it is strained. (van Wyk and Wink, 2004)

Tincture: (*tincture*) this refers to an alcoholic solution (usually containing 30 to 70% water) prepared from medicinal plant material. This herbal mixture is extracted for a specified period, after which it is pressed and/or strained to separate the liquid and solid material. A mother tincture is often prepared by using 70% ethanol, and the solution is then diluted with clean water to a predetermined yield. (van Wyk and Wink, 2004)

Ointments, pastes or gels: these are semisolid preparations intended for external application that contain medicinal substances in a suitable carrier substance (water or oily solvents). (van Wyk and Wink, 2004)

Inhalations: they are liquid preparations containing volatile ingredients which when vaporised in a suitable manner, are intended to be brought into contact with the lining of the respiratory tract. The ingredients must be volatile at room temperature, where they might be inhaled from an absorbent pad on which they have been placed, or they may need to be added to water heated to about 65°C, but not boiling water, and the vapour inhaled for 5-10 minutes. (van Wyk and Wink, 2004).

Snuffs: they are preparations of finely powdered, dried medicinal plants that can be drawn up into the nostrils through inhalation. (van Wyk and Wink, 2004).

May God have mercy on those who lead the way
and those who come behind, and those who fulfil their vows,
and those who seek to fulfil them,
with His Grace and bounty, His great benefits and favours!
For He is the best object of petition and the noblest object of hope;
And God is the best protector and the most merciful of those who show mercy, *
and the best of friends and the best of heirs
and the best replacer of what has been consumed
and provider for those devoted
who sow and till the soil of good works.
And God bless Muhammad and all the Prophets and Messengers!
Amen, O Lord of created beings!

[*Mathnâwi* IV, Prologue] in Jewels
of Rememberance, Rumi.

* Qur'an: Surah Yusuf (Joseph), 12:64.

Chapter 1
Introduction

1.1 Traditional medicine

Approximately 80% of the world's population is dependent on traditional medicine (Owalabi et al., 2007; van Wyk et al., 2009). The World Health Organisation (WHO, 2002) has described traditional medicines as "diverse health practices, approaches, knowledge and beliefs incorporating plant, animal and/or mineral based medicines, spiritual therapies, manual techniques and exercises applied singularly or in combination to maintain well-being, as well as to treat, diagnose or prevent illness" (WHO, 2002). This definition encompasses traditional medicine throughout the world and indicates that the use of medicinal plants is only a small component of a more integrated and holistic approach to health care. However, a general definition does not adequately explain the importance and scope that traditional medicine and traditional healers have on the African continent.

From comparisons between Chinese medicines and Ayurvedic treatment, it is evident that many practices have been exchanged between each of these methods of healing (Swerdlow, 2000). These methods have been used for generations and over time have evolved with contributions that can be found in Sri-Lanka, Thailand and Africa (Swerdlow, 2000). These two systems include using one plant for many conditions or many plants to treat just one ailment and remedies are still the mainstay of treatment in the less commercialized and developed areas of India and China, where they continue to show their worth as becoming more popular in the western world (Swerdlow, 2000). The successes that these two ancient medicinal practises have had are not unique as the same principles are applied to traditional healers in Africa. This system has also survived through many generations and has been passed on by teachers, parents and mentors.

Traditional healers are widely referred to as medicine men or "shaman". These terms are used to denote a person who is skilled in the use of plants and other natural substances and in the rituals of medicine and healing (Swerdlow, 2000). In South Africa, traditional healers are known, most commonly, as "inyanga" and "isangoma" (Zulu), "ixwele" and "amaquira" (Xhosa), "nqaka" (Sotho), "bossiedokter" and "kruiedokter" (Afrikaans) (Eloff and McGaw in Ahmad et al., 2006). There are an estimated 200 000 indigenous traditional healers in

South Africa and approximately 60% of the population consult these healers (van Wyk et al., 2009). This may be due to their large numbers and accessibility as well as the familiarity they have with their patients. As in Western medicine, the variety of different ailments and treatments lends itself to the need for specialization. Thus, under the label of traditional healer, there are herbalists, herb sellers, traditional birth attendants, bone setters, diviners, faith healers, traditional surgeons, spiritualists and many others (Evans, 2002). The fact that traditional healers are an important source of primary health care in Southern Africa is supported by the WHO (2002). The cultural importance and high botanical density in the region are the most likely reasons why the demand is so high (McGaw et al., 2005; Light et al., 2005; Lewu and Afolayan, 2009). Despite the well known fact that traditional medicine is a diverse and often intricate form of health care, its basis is that of holistic healing. All traditional healers treat the psychological as well as physical aspects of each patient. This ensures an all round state of health and wellbeing. The physical symptoms are treated using various medicinal plants alone and in combination. Different parts of these plants may have different effects as they often contain different active ingredients, therefore sometimes the whole plant may be used while at other times only a specific part (van Wyk and Wink, 2004).

1.2 Medicinal plants

The traditional medicines that are generally used by the majority of the patients are mostly administered by traditional healers and largely consist of crude plant material and extracts (Reid et al., 2005). In South Africa, as in other developing countries of the world, traditional medicine still forms the backbone of healthcare (Light et al., 2005). The indigenous population, specifically, rely on traditional medicine for all aspects of primary health care (Grierson and Afolayan, 1999; Kelmanson et al., 2000; van Wyk et al., 2009).The use of medicinal plants by the people of South Africa dates back to the early settlement of the native Hottentots. They used many plant species to treat different diseases, (Stone, 1764; Maclagan, 1876; Neuwinger, 2000; Lewu and Afolayan, 2009).

Medicinal plants tend to normalize physiological function and correct underlying causes of a disorder instead of temporarily alleviating symptoms (Murray and Pizzorno, 1999; Vermani and Garg, 2002). Generally, medicinal plants are used in two ways. They are sources of biologically important or active compounds (pure, chemically defined active principles) or as

an element in complex mixtures containing a broad range of constituents e.g. essential oils, tinctures, or extracts (Hamburger and Hostettman, 1991; Neuwinger, 2000).

The modes of administration as well as preparation methods need to be taken into account when studying these medicines. This includes the quantities, the addition of solvents, as well as preparation procedures such as boiling or burning (van Wyk et al., 2009). These vary widely as different parts of the plant are eaten, drunk as teas, or mashed in water for compresses or poultices when freshly harvested. Dried herbs can be soaked in liquids for the same use but are frequently crushed to powder and made into ointments by mixing with fats. These can be massaged into skin scratches or wounds (von Koenen, 2001).

1.3 Combination therapy

Even though efficacies between extracts (Kang et al., 1992), essential oils (Lachowicz et al., 1998), and combinations of medicinal plants with conventional antimicrobials (Scott et al., 1995; Shin, 2003; Filoche et al., 2005) have been studied, these have not been done in great depth. Williamson (2001), in a synergy review, has stressed the need for further interactive investigations. This is important in the study of plants such as *A. afra* as most remedies using such plants are prepared using one or two others together and if studied could prove to have a synergistic effect.

Moreover, evidence for synergy or antagonism between three or more plant combinations is sparse (van Vuuren and Viljoen, 2011). Thirty years ago Rahal, (1978) mentioned this fact and until today, not much information has been gathered. When considering all the traditional health systems it becomes apparent that an enormous gap exists between where we are currently looking for new remedies (synthesizing new entities) and where we should be looking i.e. to the tried and tested remedies of old.

“Synergy is what happens when you take two components, combine them and the resulting combination is more potent than the individual components. The result is not just double but many times their singular effect” (Dugmore and van Wyk, 2008). Synergy is defined as the result of the combined action of constituents being greater than expected from a collection of individual contributions (Williamson, 2001). It is important to distinguish between synergy

and polyvalent action as the latter is merely the various effects that multiple active constituents produce when used in combination (Williamson, 2001).

One of the main reasons for the interest in synergy, by combining two or more plants with antimicrobial activity together, is the need to delay or prevent the increasing emergence of resistant strains of highly pathogenic micro-organisms. The rational for this is that the likelihood of micro-organisms to develop resistance to all the antimicrobials in a combination is low. An example of this in conventional medicine is when treating Tuberculosis; a multiple drug therapy is used to reduce the risk of infection with resistant strains. Another contributing factor is that many antimicrobials produce an unacceptable level of toxicity when used in high doses. Thus, the use of multiple low dose antimicrobial agents is beneficial in reducing the side effects of single high dose compounds (Eliopoulos and Moellering, 1996). Other advantages include the treatment of broad spectrum infections. Among them are Gram-positive, Gram-negative, mycobacterium, yeasts and moulds, all of which can be targeted at the same time.

In the ethnobotanical studies of various plants, many publications have noted that synergy is often dependant on the organism tested as well as the doses that are used (Kamatou et al., 2006; van Vuuren et al., 2009; Suliman et al., 2010).Wagner and Ulrich-Merzenich (2009) are of the opinion that whole plant extracts have improved effectiveness as compared to the single constituent isolated from the plant, but due to the complexity of the constituents, it is difficult to identify which component/s are responsible for their synergy (Williamson, 2001). Traditional medical disciplines such as South African traditional healers, Chinese medicine, and Ayurveda, use combinations of different plants to treat various ailments. It is important, therefore, to establish whether the various plants used by traditional healers act in synergy, additively or antagonistically when combined.

It is known that medicinal plants have been used since antiquity (Bodeker et al., 1999; Rios and Recio, 2005) to treat common infectious diseases and it has been long acknowledged that essential oils exhibit antimicrobial properties (Finnemore, 1926; Al-Bayati, 2008). Moreover, when using a mixture or combination of plants many more compounds are present and may act at multiple target sites, and therefore may act synergistically to increase effectiveness (Adwan et al., 2006; Al-Bayati, 2008).

The major aim of many *in vitro* testing procedures with traditional medicinal plants is that the focus is on the identification of a single compound. Traditional healers, however, are of the opinion that this is a "reductionist" approach. They argue that this approach misses the synergism or interactive effects of the multiple ingredients in a complex mixture (Bodeker et al., 1999). Combinations are frequently used in traditional medicine because of the suspected synergism that takes place among the phytochemicals used (Mythilypriya et al., 2008) as well as the aim at curing several diseases simultaneously (Abu-Shanab et al., 2004).

Plants may be combined with other components such as animal or insect parts (Reyneke, 1971; Louw et al., 2002), other medicinal plants (Watt and Breyer-Brandwijk, 1962; Hutchings et al., 1996; Felhaber, 1997), and adjuncts such as milk, salt, honey, vinegar, or brandy (Watt and Breyer-Brandwijk, 1962). These combinations are used in order to yield more potent and safer medicines (Reyneke, 1971;; Shin, 2003; Pyun and Shin, 2006). These adjuncts are still used in traditional medicine today, although combinations with other plants have proved to be the most valuable in the treatment of ailments (Louw et al., 2002).

The rationale for using plant combinations is the desire to produce a combination that has multiple mechanisms of action, and that the expected action of the combination would be greater than the sum total of the individual or known and unknown chemical components (Harris, 2002). The possibility of other interactions i.e. additivity, indifference or antagonism should also be considered (van Vuuren and Viljoen, 2011).

Different plant species in combinations have been scientifically validated. Examples include the commercially marketed combination of *Ginkgo biloba* with Echinacea wherein synergistic interactions are present (Williamson, 2001). In another study, the combination of *Cinnamomum cassia* with *Allium tuberosum* and *Cornus officinalis* was tested in different ratios. The ratio 8:1:1 (*C. officinalis*: *C. cassia*: *A. tuberosum*) possessed antimicrobial efficacy over a wide range of micro-organisms (Hsieh et al., 2001). *Eucalyptus dives* and *Coriandrum sativum* were combined to determine the food preservation efficacy against 12 organisms (Delaquis et al., 2002). Synergy was obtained against *Yersinia enterocolitica*. Another study combining the essential oils of tea tree with lavender and tested against two dermatophytes. Various ratios of the combination were tested and isobolograms were plotted. The results demonstrated antimycotic activities (Cassella et al., 2002). Kamatou et al. (2006) investigated the combination of the extracts of *Salvia chamelaeagnea* with *Leonotis leonurus*

to treat respiratory infections. Synergy was observed against the Gram-positive bacteria while antagonism, synergy and/or additive interactions were observed against the Gram-negative bacteria. Rosemary and clove essential oils were combined in the study wherein MIC assays as well as time-kill assays were employed (Fu et al., 2007). Additive interactions were noted using MIC ratios against *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Proteus vulgaris* and *Pseudomonas aeruginosa*. Time-kill studies revealed only concentrations twice that of the MIC values produced a cidal effect. The traditional use of the combination of *A. afra* with *L. javanica* essential oil used in traditional African medicine to treat *K. pneumoniae* related respiratory tract infections was validated by utilising a time-kill assay (van Vuuren, 2007). Al-Bayati, (2008) tested the essential oils and the methanol extracts of the combination of *Thymus vulgaris* with *Pimpinella anisum* (Iraqi folk medicine) against nine pathogens. Additive interactions were noted for this combination. Gutierrez et al. (2009) tested the essential oils of *Origanum vulgare* with *Thymus vulgaris* and found additivity against food spoilage bacteria. These studies provide some credibility towards the uses of plant combinations for enhanced antimicrobial efficacy.

1.4 Respiratory tract conditions

It is commonly noted that traditional healers prescribe the use of plants singularly and in combinations for respiratory infections (Watt and Breyer-Brandewijk, 1962). Therefore, in an effort to improve efficacy of traditional medicines, this study was undertaken in order to establish a scientific basis for the use of the medicinal plants in combination for the treatment of respiratory infections. Respiratory Gram-positive pathogens responsible for infections include *Bacillus cereus*, *Enterococcus faecalis*, *Mycobacterium smegmatis*, *Staphylococcus aureus* and *Streptococcus pyogenes*. Gram-negative pathogens associated with respiratory infections include *Klebsiella pneumoniae*, *P. aeruginosa*, and *Moraxella catarrhalis*. Some fungi that cause respiratory infections are *Cryptococcus neoformans*, *Aspergillus niger* and *Alternaria alternate* (van Vuuren, 2007). Details of some the most frequently used test pathogens in this study are given as follows.

M. catarrhalis is Gram-negative diplococci and is considered an important cause of upper and lower respiratory tract infections in children and adults. It is reported to be the single cause of sinusitis, otitis media, tracheitis, bronchitis and pneumoniae in children (Verduin et al., 2002). *M. catarrhalis* is also a common cause of nosocomial infections and causes

laryngitis, bronchitis and pneumoniae (Verduin et al., 2002). It is the third most common pathogen after *Haemophilus influenza* and *Streptococcus pneumoniae* causing lower respiratory tract infections in adults (Nicotra et al., 1986; Chin et al., 1993).

Klebsiella pneumoniae is a Gram-negative encapsulated bacteria, an opportunistic pathogen and causes septicaemia, pneumonia, urinary tract infection, as well as soft tissue infections. It is a prominent nosocomial pathogen causing respiratory tract infections (Podschun and Ullmann, 1998; Brisse et al., 2009). It is one of the leading causes of community-acquired pneumoniae in some countries like South Africa (Ko et al., 2002; Yu et al., 2007; Brisse et al., 2009).

E. faecalis is a Gram-positive pathogen and causes up to 90% of enterococcal infections in humans (Kayaoglu and Ørstavik, 2004). It most commonly infects the urinary tract, endocardium causing endocarditis and the abdomen (Boyd and Hoerl, 1981; Jett et al., 1994). It can also infect the lungs, soft tissues, paranasal sinuses and the ears (Rantz and Kirby, 1943; Horvitz and von Graevenitz, 1977; Berk et al., 1983; Maki and Agger, 1988; Doyle and Woodham, 1991; Graninger and Ragette, 1992; Bonten et al., 1993; Jett et al., 1994)

Cryptococcus neoformans is an encapsulated yeast, which enables it to reduce size in certain environments, which is thus then ideal for alveolar deposition following inhalation of the pathogen. It is a major pathogen in the immune-compromised patient (causing cryptococcosis) with underlying conditions such as Acquired Immune Deficiency Syndrome (AIDS), lymphoma and patients undergoing corticosteroid therapy (Nielson et al., 1977; Perfect, 1989; Levitz, 1991; Cherniak and Sundstrom, 1994; Diamond, 1995; Hogan et al., 1996; Nosanchuk et al., 2000). It causes asymptomatic pulmonary infections followed by meningitis. When limited to the lungs it causes pneumonia (King and DeWitt, 2009

S. pneumoniae is an alpha haemolytic Gram-positive cocci. Acute respiratory tract infections are often caused by *S. pneumoniae*, the main aetiological agent. It is exclusive to humans and is one of the most common community-acquired respiratory tract infections requiring antimicrobial therapy. It is also a leading cause of bacterial pneumoniae (File, 2000; Bessen, 2009; WHO, 2009; Farrel et al., 2010). *S. pneumoniae* causes diseases such as bacteremia, meningitis, pharyngitis, rhinitis, otitis, sinusitis, arthritis and pneumonia (Demachy et al., 2001; Warda et al., 2009).

S. pyogenes is referred to as the large colony-forming beta-haemolytic group (Bessen, 2009). It is the causative agent of the "strep" throat representing pharynhitis and tonsillitis infections. It is also a cause of non-bulbous impetigo in children in underdeveloped countries. Invasive *S. pyogenes* causes an autoimmune disease Rheumatic fever, with serious cardiac manifestations (Carapetis et al., 2005; Bessen, 2009).

Streptococcus agalactiae is a Gram-positive group B streptococcus and is a commensal normal colonizer of the vagina, gastrointestinal and upper respiratory tract of healthy humans. It causes severe disease such as pneumonia, skin and soft tissue infections, septic arthritis and meningitis in immuno-compromised animals and humans (Bessen, 2009; Woods and Levy, 2010).

M. smegmatis is a Gram-positive, rapidly growing mycobacterium. It was selected for this study due to its genetic similarity to *Mycobacterium tuberculosis* and its lack of virulence as an infectious organism (Mitscher and Baker, 1998). *M. smegmatis* has been associated with soft tissue or wound infections as well as respiratory tract infections (Vonmoos et al., 1986; Newton et al., 1993; Cox et al., 1994; Pennekamp et al., 1997; Ergan et al., 2004).

1.5 Plants used in treating respiratory tract infections

Focussing on combinations used for respiratory infections, numerous ethnobotanical remedies are administered. Many of these have not been validated scientifically. A compilation of the ethno-botanical uses of the combinations used for respiratory infections in Southern Africa has been reported and some are summarised in Table 1.1.

On examination of these combinations, it is clear that *A. afra* is predominantly administered in combination. Hence, this study focuses on validating the antimicrobial efficacy of those combinations containing *A. afra*.

Table 1.1 Plant combinations used for the treatment of respiratory infections.

Plants in combination	Uses	Administration	Reference
A. afra with *Eucalyptus globulus*	Respiratory complaints	Crushed leaves or steam from infusions are inhaled or decoctions are taken	Watt and Breyer-Brandwijk, (1962); Hutchings et al. (1996).

Plants in combination	Uses	Administration	Reference
A. afra with *Agathosma betulina*	Respiratory complaints	Herbal wine	Watt and Breyer-Brandwijk, (1962).
A. afra with *Zanthoxylum capense*	The Europeans and Africans use it in febrile conditions, and it is used as a treatment for colds	A decoction and an infusion of the leaf is used	Watt and Breyer-Brandwijk, (1962).
A. afra with *Osmitopsis asteriscoides*	Respiratory complaints	Tincture	Watt and Breyer-Brandwijk, (1962).
A. afra, Eucalyptus globulus with *Leonotis microphylla*	Fever, chest infections and digestive disturbances	Infusion	Watt and Breyer-Brandwijk, (1962).
A. afra, Zanthoxylum capense with *Allium sativum*	Respiratory complaints	Decoction	Watt and Breyer-Brandwijk, (1962).
A. afra with *Lippia javanica*	Fevers, respiratory complaints, measles and as a prophylactic against lung inflammations	Infusion, taken with milk	Watt and Breyer-Brandwijk, (1962).
A. afra, Osmitopsis asteriscoides with *Eucalyptus globulus*	Respiratory complaints	Infusion, tincture	Watt and Breyer-Brandwijk, (1962); van Wyk et al. (2009).
A. afra with *Tetradenia riparia* and salt	Coughs	Decoctions	Hutchings et al. (1996).
A. afra with *Alepidea amatymbica*	Colds and flu	Leaves and root/rhizome	Jäger, 2003.
A. afra with *Warburgia salutaris*	Acute bronchitis, coughs from colds or flu, fever	Leaves and bark	Felhaber, (1997); Jäger, (2003).
A. afra, Alepidea amatymbica with *Leonotis leonurus*	Asthma	Leaves, root and leaves	Felhaber, (1997).
A. afra, Warburgia salutaris with *Acorus calamus*	Chronic bronchitis and emphysema	Leaves, bark and rhizome	Felhaber, (1997).
A. afra, Ruta graveolens with *Pteronia divaricata*	Fever and colds	Unknown	Hulley et al. (2010).

Plants in combination	Uses	Administration	Reference
Cheilanthes hirta with *Mohria cafforum*	Colds and sore throats in restless children	Burned together and taken in as an inhalation	Hutchings et al. (1996).
Phoenix reclinata, Euclea natalensis, Capparis tomentosa with *Maytennus heterophylla*	Pleurodynia and pleurisy	Steam from the decoction is blown into the wound	Hutchings et al. (1996); Bryant, (1966).
Cannabis sativa with *Warburgia salutaris*	Dry cough, asthma	Powdered bark of *Warburgia salutaris* and leaves of *Cannabis sativa* are mixed and smoked	Hutchings et al. (1996); Bryant, (1966).
Nymphaea nouchali with *Tulbaghia* species	Coughs and colds	Decoctions	Hutchings et al. (1996).
Ranunculus multifidus with *Helichrysum nudifolium*	Severe coughs and sore throats	Milk infusions of roots	Hutchings et al. (1996).
Capparis brassii, Zanthoxylum capense, Capparis tomentosa with what is reported to be *Cyrtanthus obliquus* but is probably a *Eucomis* species	Chronic coughs	Decoction	Hutchings et al. (1996); Bryant, (1966).
Ozoroa paniculosa with *Berchemia zeyheri*	Acute inflammatory conditions of the chest	Taken by mouth	Hutchings et al. (1996).
Pterocelastrus echinatus with *Alepidea amatymbica*	Respiratory ailments	Decoction	Hutchings et al. (1996); Pujol, (1990).
Cucumis hirsutus with *Aster bakeranus*	Chronic coughs	Decoctions	Hutchings et al. (1996); Bryant, (1966).
Berkheya raphontica with *Athrixia phylicoides*	Dry hacking coughs	Decoctions	Watt and Breyer-Brandwijk, 1962; Hutchings et al. (1996).
Eucalyptus globulus, Corbichonia decumbens with *Acorus calamus*	Blocked nose	Boiled infusion	Felhaber, (1997).
Warburgia salutaris with *Acorus calamus*	Pneumonia	Infusion	Felhaber, (1997).
Lippia javanica with *Aloysia triphylla*	Colds, fever, asthma	Herbal teas	van Wyk and Wink, (2004).

1.6 *Artemisia afra* (previous studies)

A. afra is known to be widely used for medicinal purposes as well as being one of the oldest medicinal plants used in South African traditional medicine (Mangena and Muyima, 1999; Thring and Weitz, 2006). It is considered a future flagship of traditional medicine due to its broad spectrum of activity (Mangena and Muyima, 1999; Liu et al., 2009; van Wyk et al., 2009).

A. afra has been documented in the literature for its chemistry, cultivation, toxicology, and quality control. As depicted in Figure 1.1, the bioactivity (53%) of *A. afra* has been the focus of a majority of studies. Antioxidant (Graven et al., 1992; Burits et al., 2001), antimalarial (Weenen et al., 1990; Kraft et al., 2003; Clarkson et al., 2004; Gathirwa et al., 2007), anti-nematodal (McGaw et al., 2000), cardiovascular (hypotensive) (Guantai and Addae-Mensah, 1999), cytotoxic (Jenett-Siems et al., 2002) and sedative (Nielsen et al., 2004; Stafford et al., 2005) effects have been demonstrated and well documented (van Wyk, 2008a).

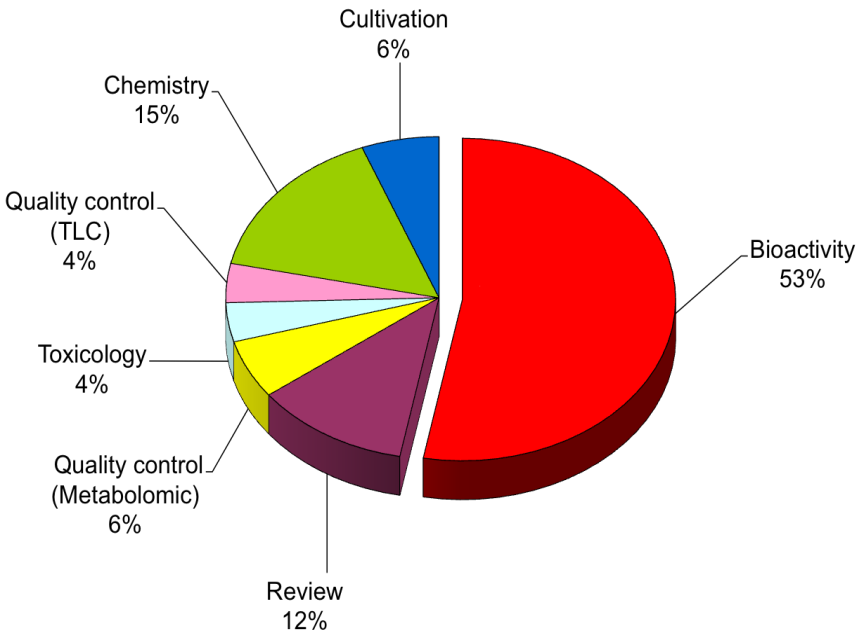


Figure 1.1 The relative percentage of research by topic published on *A. afra.*

Furthermore, Figure 1.2, which examines the literature pertaining specifically to the biological activity, shows that most of the literature thus far available regarding *A. afra*'s biological activities have focussed on antimicrobial aspects.

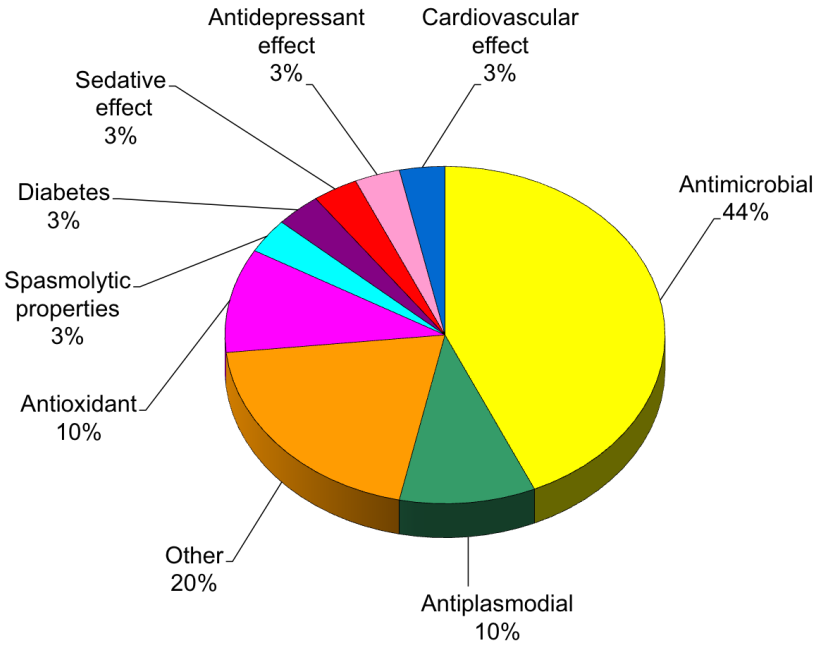


Figure 1.2 The relative percentages of research by bioactivity published on *A. afra.*

Some studies conducted on the antimicrobial activity of *A. afra* include Graven et al. (1992), Gundidza, (1993); Bruneton, (1995); Rabe and van Staden, (1997); Mangena and Muyima, (1999); Huffman et al. (2002); Muyima et al. (2002); Jäger, (2003); Motsei et al. (2003); van Vuuren and Viljoen, (2006); Viljoen et al. (2006b), Vagionas et al. (2007); van Vuuren, (2007). *A. afra* combinations, however, have been neglected. Further information regarding the botanical description, medicinal uses, geographical locality, chemistry of *A. afra* and other plants examined in this study may be found in Appendix A3.

1.7 *A. afra* in combination

When examining the research undertaken on *A. afra* using the Google search engine, "*Artemisia afra*" gave 16000 results (7/01/2011). When examining this more closely by using the terminology "combination", 1860 results emerged http://www.google.co.za/search?hl=en&biw=1280&bih=683&q=Artemisia+afra&as_q=combination&btnG=Search%C2%A0within%C2%A0results). A Pubmed search on "*Artemisia afra*" yielded 24 results. Limiting the search to "*Artemisia afra* in combination" yielded zero results. Using ScienceDirect, a search on "*Artemisia afra*" alone yielded 148 articles and when limited to "*Artemisia afra* in combination", 65 were identified. On Scopus, 70 results were found for "*Artemisia afra*" alone and for "*Artemisia afra* in combination", only nine results were obtained. (6/01/2011).

This lack of information shows that there is a need for scientifically valid research on this subject.

Some instances where plants have been combined with *A. afra* for the treatment of microbe related infections have been documented in the ethnobotanical literature and are included in Table 1.1. Accounts of the administration of *L. javanica* with *A. afra* to treat respiratory disorders have been reported (Hutchings et al., 1996; Watt and Breyer-Brandewijk, 1962). *A. afra* has also been noted to be used either alone or in combination with the leaves of *Eucalyptus* spp. by the Xhosa, as an influenza remedy (Watt and Breyer-Brandewijk, 1962). Watt and Breyer-Brandewijk, (1962) sites many examples in which *A. afra* has been used in combinations with common household herbs e.g. ginger, thyme, rosemary, mint and chamomile. More specialised to traditional healers, as they are widely available and indigenous to the country of South Africa, are combinations involving species such as *O. asteriscoides* and *E. globulus* (Watt and Breyer-Brandewijk, 1962). Decoctions of the leaves of *A. afra* and *Agrimonia bracteata* are used for colds and decoctions of *T. riparia* and *A. afra* with salt are used to treat coughs in Southern Africa (Hutchings et al., 1996; Liu et al., 2009). These combinations sometimes involve two or sometimes three plants. Other examples of combinations with *A. afra* is the use of *A. afra*, *R. graveolens* and *P. divaricata*, to treat fever and colds (Hulley et al., 2010) other examples include, *A. afra* leaves and *W. salutaris* bark for the treatment of cough from cold or flu as well as acute bronchitis (Felhaber, 1997). A combination of *A. afra* leaves and *A. amatymbica* root is drunk for colds and flu. Similarly, steam inhalation therapy is used with the combination of *A. afra* and *A. amatymbica* rhizome. Asthma is treated with a combination, which is drunk, of *A. afra* leaves, *A. amatymbica* root and *L. leonurus* leaves (Felhaber, 1997). A combination of *A. afra* leaves, *W. salutaris* bark, and *A. calamus* rhizome is used to treat chronic bronchitis and emphysema (Felhaber, 1997).

A. afra in combination with other plants in double combination and in triple combinations were analyzed in this study to determine the antimicrobial interactions present. Figure 1.3 shows the double combinations that are tested along with the method of administration. Figure 1.4 shows the triple combinations of *A. afra* that are evaluated.

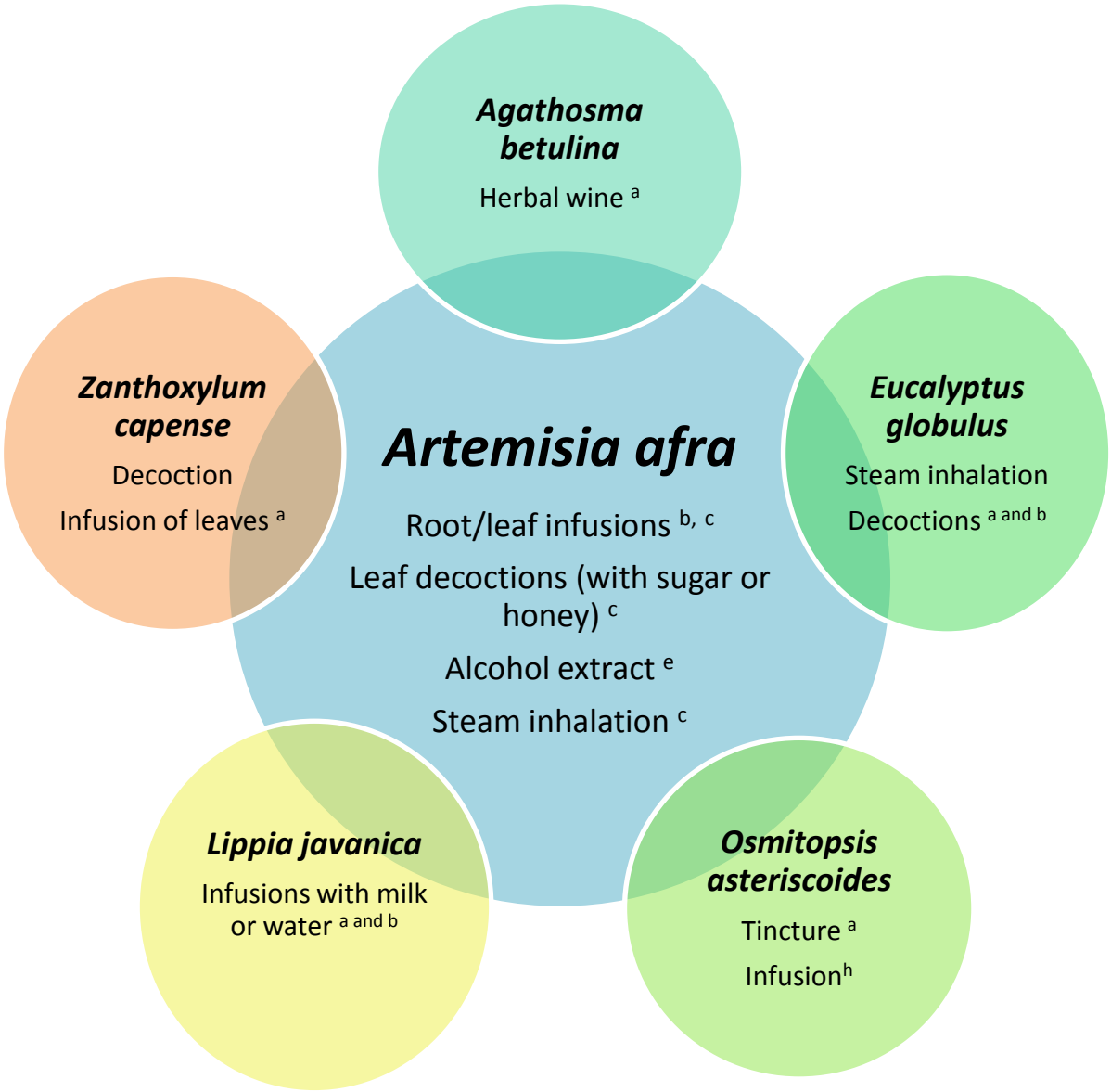


Figure 1.3 *A. afra* in double combinations. (Watt and Breyer-Brandewijk, 1962[a]; Hutchings et al., 1996[b]; van Wyk et al, 2009[c]; van Wyk and Wink, 2004[d]; von Koenen, 2001[e]; Felhaber, 1997[f]; Moolla, 2006[g]; Scott and Springfield, 2004[h]).

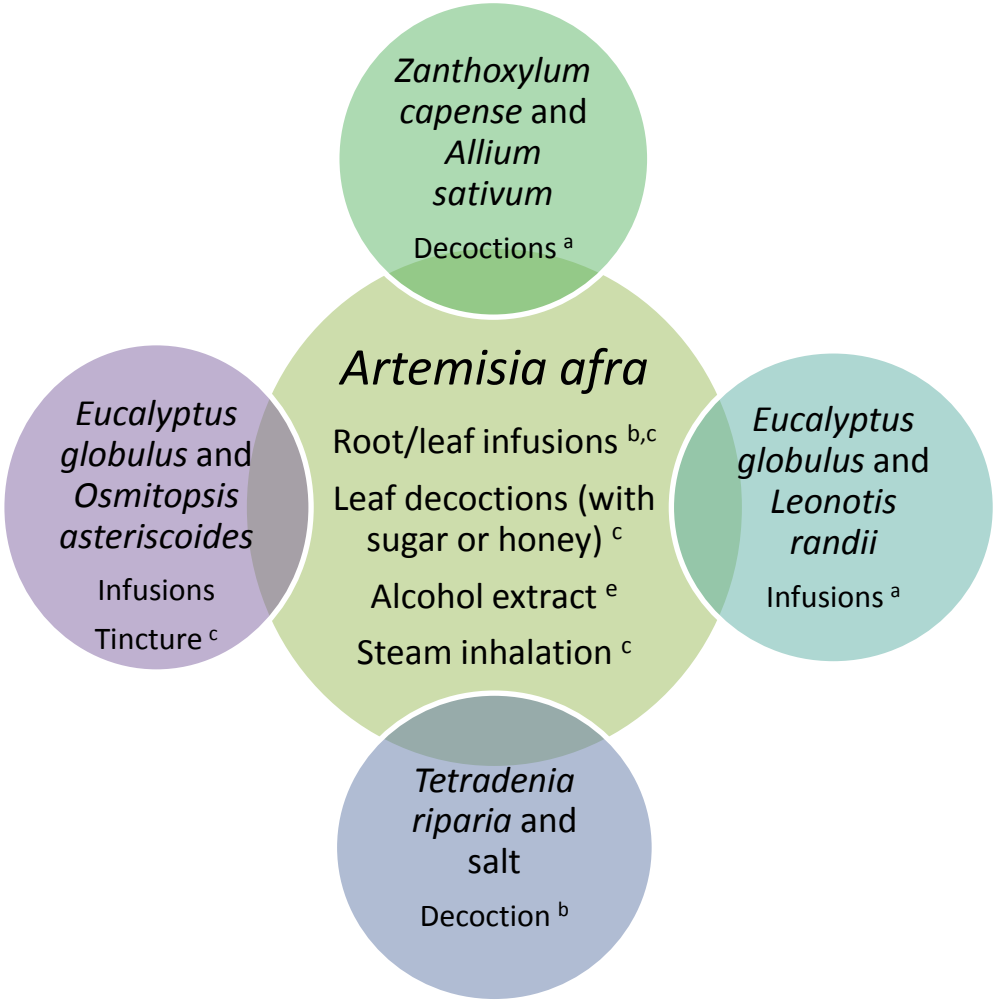


Figure 1.4 *A. afra* in triple combinations. (Watt and Breyer-Brandewijk, 1962[a]; Hutchings et al., 1996[b;] van Wyk et al, 2009[c]; van Wyk and Wink, 2004[d]; von Koenen, 2001[e]; Felhaber, 1997[f]; Moolla, 2006[g]).

1.8 Aims and objectives of the study

This study was undertaken with the aim of finding a scientific rationale for the antimicrobial use of *A. afra* in combination. A further breakdown of objectives for this study is as follows:

1. To identify plants used in combination with *A. afra* by examination of the ethnobotanical literature.
2. To extract (dichloromethane: methanol and aqueous extracts) and distil collected plant material.
3. To perform gas chromatography coupled with mass spectrometry (GC-MS) on the essential oils obtained from hydrodistillation.
4. To perform thin layer chromatography analysis on the plants essential oils independently and in combination with *A. afra*.

5. To carry out antimicrobial screening using minimum inhibitory concentration (MIC) assays on essential oils and extracts.
6. To perform interactive combination studies comparing data obtained from the initial screening antimicrobial studies i.e. with combinations utilizing fractional inhibitory concentration (∑FIC) studies and isobologram interpretation.
7. To investigate the use of adjuncts in combination with *A. afra* using MIC and ∑FIC assays.

Chapter 2

Materials and Methods

2.1 Collection of plant material

Plant species (Table 2.1) were collected (Figure 2.1) at various localities during the summer months between the periods January 2007-April 2007. All plant identities were confirmed by a qualified botanist, either Andrew Hanky or Professor Alvaro Viljoen. The various species and their collection localities are shown in Table 2.1. Voucher specimens are deposited in the Department of Pharmacy and Pharmacology, University of the Witwatersrand.

Table 2.1 Collection data of the plants studied.

Voucher	Species	Locality	Material distilled (g)	Essential oil yield (%w/v)
SFVV13	*Agathosma betulina*	Landmeterskop, Middelberg (Mpumalanga)	955	0.9
SFVV14	*A. afra*	Klipriviersburg (Gauteng)	5902.7	0.6
SFVV67				
SFVV33	*E. globulus*	Cresta (Gauteng)	3823.4	0.8
SFVV74				
SFVV46	*L. randii*	Walter Sisulu Botanical Gardens, Johannesburg (Gauteng)	117.6	No oil
SFVV8	*L. javanica*	Fairlands (Gauteng)	2893.3	0.2
SFVV70				
SFVV23	*O. asteriscoides*	Hermanus, Cape Town	2233.2	0.8
SFVV47	*T. riparia*	Walter Sisulu Botanical Gardens (Gauteng)	6871.7	0.1
SFVV62				
SFVV72				
SFVV76				
SFVV48	*Z. capense*	Walter Sisulu Botanical Gardens (Gauteng)	262.2	No oil

2.2 Sample preparation

2.2.1 Dichloromethane: methanol extracts

Extracts were prepared by submerging the dried macerated plant material (±50 g) in a 1:1 mixture of dichloromethane and methanol (CH_2Cl_2: MeOH) (Merck). This was heated to 37°C for 24 hrs in a water bath. Thereafter, the solution was filtered using cotton wool. The filtered solution was then evaporated by air-drying in a fume hood. The resulting extract was then stored at 4°C for antimicrobial analysis.



Figure 2.1 Collection of *A. afra* in Klipriviersburg.

2.2.2 Aqueous extracts

Aqueous extracts were prepared by submerging macerated plant material (±50 g) in sterile distilled water, which was then kept in a water bath at 37°C overnight. Thereafter, it was filtered using Wattman® No 1 filter paper and stored at -80°C for 48 hrs before lyophilisation (van Vuuren and Viljoen, 2006).

2.2.3 Distillation of plant material

For the aromatic plants, the essential oils were isolated by conventional hydro-distillation in a Clevenger-type apparatus. A round bottomed flask was packed with fresh leaf material (±1000 g) (Figures 2.2 and 2.3) and ±500 ml of distilled water was added. The flask was then sealed and the cooling system was set up. Thereafter the apparatus was heated using an

electric heating mantle. The essential oils were then collected in the condenser and were decanted after three hours. The samples collected were then stored in amber vials and refrigerated for antimicrobial analysis.



Figure 2.2 Fresh leaf materials being packed into the apparatus.



Figure 2.3 Clevenger apparatus being set up.

2.2.4 Adjunct preparation

Salt, powdered skim milk, as well as powdered full cream milk was purchased from the local Checkers® supermarket. The different types of honey used i.e. orange blossom honey, wild blossom honey and blue gum honey were purchased from Woolworths®. The milk powder was made up according to the instructions indicated on the container by the manufacturer. (25.0 mg/ml). The milk was autoclaved and then allowed to cool before analysis.

The honey, brandy and vinegar were made up to a concentration of 64.0 mg/ml. The volume of acetone or water to be added was calculated according to Equation 2.2. Honey was also tested at 100% or undiluted. Salt was prepared to a concentration of 4% and diluted with water.

2.3 Chromatographic techniques

2.3.1 Thin layer chromatography

Thin layer chromatography (TLC) was done as an initial screening process to visualise the chromatographic profile of each plant. Furthermore, combinations of the plants were done in order to qualitatively determine any increase or decrease in compounds when combined.

2.3.1.1 Initial TLC screening

A preliminary screening of the plants essential oils, dichloromethane: methanol extracts and the aqueous extracts were performed in order to visualize any compounds present using TLC. In this study for the essential oils, the mobile phase in which the plates were developed comprised of toluene: ethyl acetate (93:7). The mobile phase for development of the dichloromethane: methanol extracts was methanol: water: ethyl acetate (16.5: 13.5: 100) and for the aqueous extracts methanol: water: acetone: ethyl acetate: chloroform (10:8: 30: 40: 12) was the best choice. Compounds resolved on the plate were visualized using either general or specific methods. Ultraviolet light (UV) indicates fluorescent compounds. They are examined at 365 nm (long) and at 254 nm (short) wavelength UV light (Evans, 2002). Alternatively, following development colourless compounds required a chemical reaction in order to visualize their location (Smith and Seakins, 1976). Thus, a spray reagent to produce coloured derivatives was used. For the essential oils, the plates were sprayed with an anisaldehyde-acetic-acid reagent (van Vuuren, 2007) or a vanillin-sulphuric acid reagent (Wagner and Bladt, 1996), as shown in Table 2.2. Similarly, for the extracts, vanillin-sulphuric acid reagent or natural products reagent (Table 2.2) was used (Wagner and Bladt, 1996). This required subsequent heating of the plate to approximately 110°C for 5 min to enable visualization of the compounds (Wagner and Bladt, 1996).

Table 2.2 Reagents used and its composition.

Reagent	Composition
Anisadehyde-acetic-acid	0.5 ml anisaldehyde + 10 ml glacial acetic acid + 85 ml methanol
Vanillin-sulphuric acid	(100 ml ethanol + 1 g vanillin) + (100 ml ethanol + 10 ml sulphuric acid)
Natural products-polyethylene glycol	1% methanolic diphenylboric acid + 5% ethanolic poly-ethylene glycol-4000

(Adapted from Wagner and Bladt, 1996).

2.3.1.2 High performance thin layer chromatography (HPTLC)

After troubleshooting in the initial screening of TLC and optimizing the solvents for the elution of the compounds of the essential oils, dichloromethane: methanol extracts and the aqueous extracts were determined, an automatic TLC system was used. This was performed at the Tshwane University of Technology with the assistance of co-supervisor Professor A.M. Viljoen and Joanet Maree. The plates were then developed in a CAMAG twin trough glass tank pre-saturated with the mobile phase using the CAMAG Automatic Development Chamber (ADC2). The tank was saturated for 10 min in advance and enhanced by keeping filter paper along one wall of the chamber. Each TLC plate was developed to a height of 80 mm (Paramasivam et al., 2009). All TLC runs were carried out under laboratory conditions of approximately 25°C and 39.5% relative humidity.

Sample application was performed using the CAMAG Automatic TLC Sampler 4 (ATS4) as shown in Figure 2.4 on pre-activated silica gel HPTLC plates (60GF $_{254}$, 20 × 10 cm). Samples were applied as 10 mm wide bands with N_2 flow at 15 µl/sec, positioned 10 mm from the bottom of the plate and 15 mm from the side of the plate (Paramasivam et al., 2009).

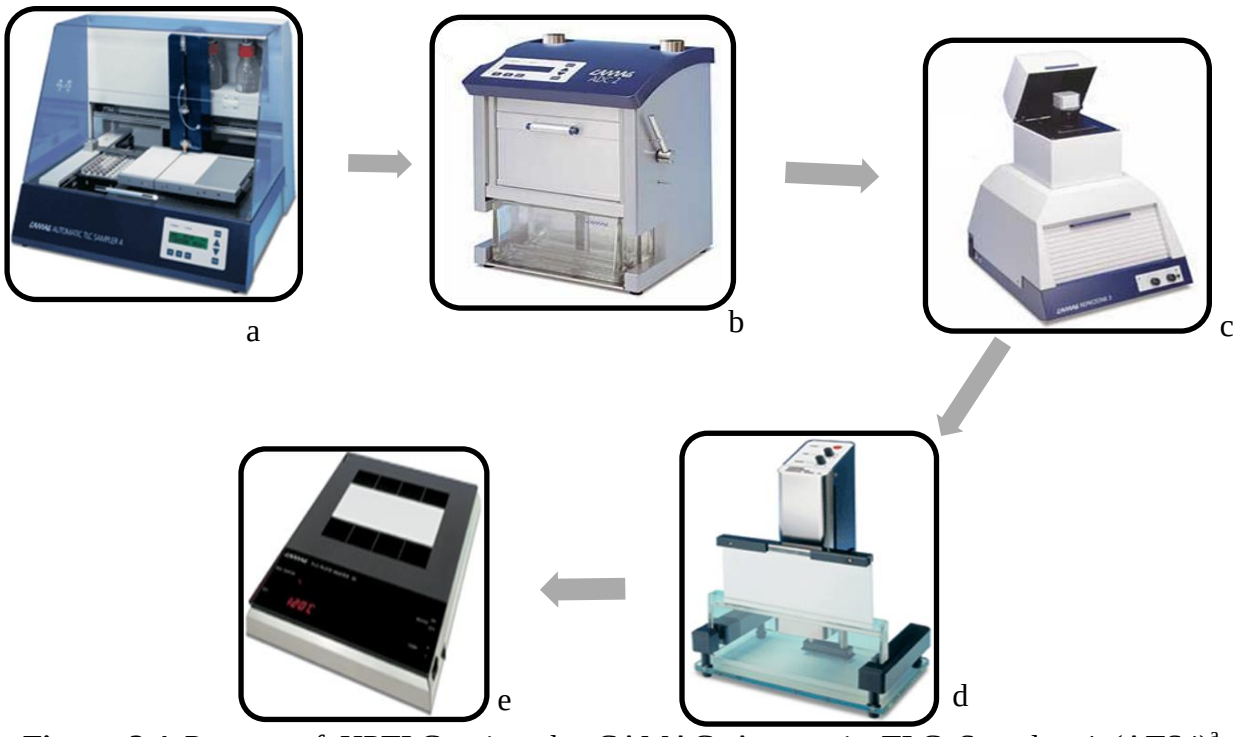


Figure 2.4 Process of HPTLC using the CAMAG Automatic TLC Sampler 4 (ATS4)[a], CAMAG Automatic Development Chamber (ADC2)[b], CAMAG Reprostar (Repro3)[c], CAMAG Chromatogram Immersion Device III[d] and the CAMAG TLC Plate Heater III[e]. (http://www.hptlc.us/v/products/application/ats4.html[a]; http://www.camag.com/v/products/development/adc2.html[b];

http://www.camag.com/v/products/assemblies/herbal_kits5.html[c];
http://www.camag.com/v/products/derivatation/derivatization.html[d]).

After development, plates were dried for five minutes, viewed, and photographed with the CAMAG Reprostar (Repro3). Plates were viewed at 254 nm and 365 nm. Images and full analysis reports are housed in the CAMAG library at Tshwane University of Technology. In order to view the individual compounds, the plates were dipped in vanillin-sulphuric acid reagent (as in Table 2.2) using a CAMAG Chromatogram Immersion Device III and then heated on a CAMAG TLC Plate Heater III at 104°C for 3-5 min. The plates were then photographed using the CAMAG Reprostar (Repro3).

2.3.1.3 Gas chromatography coupled to mass spectroscopy (GC-MS)

Essential oil samples were analyzed in order to detect the constituents present. Gas chromatography is considered an efficient method for the characterisation of essential oils (Bakkali et al., 2008; Anwar et al., 2009; Hussain, 2009). The use of gas chromatography coupled with mass spectroscopy (GC-MS) is regarded as a rapid and reliable method in order to identify individual essential oil components (Yadegarinia et al., 2006; Gulluce et al., 2007; Anwar et al., 2009; Hussain, 2009).

The oils were analysed by GC–MS (Agilent 6890N GC system coupled directly to a 5973 MS). A volume of 1 µl was injected using a split ratio (200:1) with an autosampler at 24.79 psi and an inlet temperature of 250°C. The GC system equipped with a HP-Innowax polyethylene glycol column 60 m×250 µm i.d. ×0.25 µm film thickness was used. The oven temperature program was 60°C for the first 10 min, rising to 220°C at a rate of 4°C/min and held for 10 min and then rising to 240°C at a rate of 1°C/min. Helium was used as carrier gas at a constant flow of 1.2 ml/min. Spectra were obtained on electron impact at 70 eV, scanning from 35 to 550 m/z. The percentage compositions of the individual components were obtained from electronic integration measurements using flame ionization detection (FID, 250°C). *n*-Alkanes were used as reference points in the calculation of relative retention indices (RRI). Component identifications were made by comparing mass spectra and retention indices. Library searches were carried out using NIST®, Mass Finder® and Flavour®.

2.4 Antimicrobial assays

2.4.1 Media and culture preparation

When undertaking microbial susceptibility testing, the cultures used i.e. *S. pneumoniae*, *S pyogenes*, *S. agalactiae*, *M. smegmatis*, *E. faecalis*, *M. catarrhalis*, *K. pneumoniae* and *C. neoformans* as well as the media used play an important role in the accuracy of results obtained. The culture medium selected for *in vitro* susceptibility testing must be able to support good growth of the organism to be tested. In addition, it should not have any effect on the action of the antimicrobial agent being tested (McGinnis and Rinaldi in Lorian, 1991). Table 2.3 lists the media used in the culturing of the bacteria or fungi as well as the strain numbers and characterization of the type of micro-organism. All microbiological techniques, culture and media preparation were followed as in CLSI (2003) guidelines.

Table 2.3 Media used in this study to grow bacteria and fungi.

Micro-organism strain number	Characterization of type of micro-organism	Media
S. pneumoniae ATCC 49619	Gram- positive	Mueller Hinton (Oxoid) + 2.5% sheep's blood (National Health Laboratory Services (NHLS)
S. pyogenes ATCC 8668	Gram-positive	Mueller Hinton (Oxoid) + 2.5% sheep's blood (NHLS)
S. agalactiae ATCC 55618	Gram-positive	Mueller Hinton (Oxoid) + 2.5% sheep's blood (NHLS)
M. smegmatis (clinical strain)	Gram-positive	Middlebrook–7H9-Broth (Difco^{TM}) + glycerol + Middlebrook ADC Enrichment supplement (BBL^{TM})
E. faecalis ATCC 29212	Gram-positive	Tryptone Soya broth/agar (Oxoid)
M. catarrhalis ATCC 23246	Gram-negative	Tryptone Soya broth/agar (Oxoid)
K. pneumoniae NCTC 9633	Gram-negative	Tryptone Soya broth/agar (Oxoid)
C. neoformans ATCC 90112	Yeast	Tryptone Soya broth/agar (Oxoid)

The following strains of micro-organisms were obtained from the National Health Laboratory Services (NHLS); *E. faecalis* ATCC 29212, *M. catarrhalis* ATCC 23246, *K. pneumoniae* NCTC 9633, *C. neoformans* ATCC 90112, *S. pneumoniae* ATCC 49619, *S. pyogenes* ATCC 8668, *S. agalactiae* ATCC 55618 and *M. smegmatis* (clinical strain).

2.4.2 Minimum inhibitory concentration (MIC) studies

Microbiological assays are performed in order to determine the potency or concentration of a test substance e.g. essential oil or extract to be able to determine its effects on the growth of a micro-organism (Hewitt and Vincent, 2003). A fundamental principle to these assays is that they depend on the comparison of the effect of a standard reference substance e.g. an antibiotic such as ciprofloxacin, and a sample whose potency is to be determined on a "biological system" e.g. a culture of micro-organisms. The comparison is between the reference standard and the sample of unknown potency i.e. differences in antimicrobial activity can be seen between a known antimicrobial agent and a sample that has never been tested before (Hewitt and Vincent, 2003).

One of the simplest and widely preferred techniques used to analyse the effectiveness of an antimicrobial's action is to determine the minimum inhibitory concentration (MIC) (Eloff, 1998; Hewitt and Vincent, 2003). The minimum inhibitory concentration is the lowest concentration of an antimicrobial that completely inhibits the growth of an organism (Bannister et al., 2000). It is based on the principle of contact of a test organism to a series of dilutions of test antimicrobial/substance (van Vuuren, 2007).

Two main methods are used, depending on the substrate used, agar dilution, and the broth dilution method (Hewitt and Vincent, 2003). In the agar, dilution method a serial dilution of the antimicrobial is prepared in molten agar and then plates are poured. The plates are then inoculated uniformly with about 20 µl of an overnight broth culture containing 10^5 Colony Forming Units (CFU) of a suitable test organism. After incubation the level with no growth or very few colonies are taken to be the MIC. Controls, e.g. agar plates not containing any antimicrobial as well as a reference antimicrobial with known sensitivity to the micro-organism, must be included in the study (Hewitt and Vincent, 2003).

The broth dilution method involves the growth of an organism in a liquid nutrient medium and test solutions at a series of concentrations, from reference standards as well as test substance (Hewitt and Vincent, 2003). A starting concentration of the antimicrobial is made up in broth and then two fold dilutions are serially prepared. Each test tube is then inoculated with the micro-organism of about 10^5 Colony Forming Units (CFU). After incubation, the tubes are assessed for any microbial growth. The first clear tube after turbidity is recorded as the MIC i.e. the highest dilution of the antimicrobial preventing growth is the MIC of the test

organism (Hewitt and Vincent, 2003). This technique requires large quantities of test substance, is very time consuming and when testing extracts, precipitation of the green colour made it difficult to determine the MIC (Eloff, 1998). Thus, a "sensitive" and "quick" method specifically developed to obtain MIC values for plant extracts against micro-organisms was developed by Eloff (1998). This microplate or microdilution method is not expensive, approximately 30 times more sensitive than any other method found in literature and it requires small quantities of test substance. It can be used for a large number of samples as well as a variety of different micro-organisms. Controls can be performed on the same plate at the same time and does not require a long time i.e. it is a quick and efficient method (Eloff, 1998).

All bacterial cultures were sub-cultured from stock agar plates and grown in the respective broth as shown in Table 2.3 for 24 hrs. The yeast (*C. neoformans* ATCC 90112) was incubated for 48 hrs. These were prepared using a 1:100 dilution, yielding an approximate inoculum size of 1×10^6 colony forming units (CFU)/ml). Thus, 500 µl of test organism was placed into 50 ml of sterile broth. The culture was prepared immediately before use.

2.4.2.1 Essential oil/extract and antimicrobial control preparation

Stock solutions of each of the essential oils were made to a starting concentration of 128.0 mg/ml using acetone as a diluent, and extracts were made to a concentration of 64.0 mg/ml. The volume of acetone to be added was calculated using Equation 2.1.

$$\text{Volume of acetone required (µl)} = \frac{\text{Mass of volume of essential oil/extract (mg)}}{128\text{mg/ml (essential oils) or } 64\text{mg/ml (extracts)}} \text{ x } 1000$$

Equation 2.1

Positive controls i.e. ciprofloxacin (Sigma-Aldrich) for the bacteria and amphotericin B (Sigma-Aldrich) for the yeasts were also prepared. A starting stock solution of ciprofloxacin was made up with distilled water to a concentration of 0.01 mg/ml. Amphotericin B was diluted with dimethyl sulfoxide (DMSO) initially to a concentration of 1.0 mg/ml. Thereafter distilled water was used to make up a starting stock solution of 0.1 mg/ml.

2.4.2.2 Micro-titre plate preparation

Under aseptic conditions (laminar flow unit), 100 µl of sterile water was added to each well of a 96 well micro-titre plate. A negative control (a) i.e. 100 µl of sterile water was added in row A12 (Figure 2.6) and 100 µl of conventional antimicrobial (b) (ciprofloxacin for the bacteria and amphotericin B for the yeasts) was placed into well A1 (as shown in Figure 2.5), as positive controls. One hundred micro-litres of each of the prepared test plant samples dissolved in water/acetone were placed in the respective wells in row A. These wells were labeled as depicted in Figure 2.5. Serial dilutions were then done in a vertical orientation, from A to H, using a multi-channel pipette. Thereafter, micro-titre plates were removed from the laminar flow unit and 100 µl of prepared culture was placed into each if the 96 wells. Micro-titre plates were sealed with sterile adhesive sealing film (AEC Amersham) to prevent evaporation of the essential oils. The remaining prepared culture was incubated along with another negative control, which was a streak plate of the culture used in the assay. This was carried out in order to determine the purity of the culture by the identification of single identical colonies. The micro-titre plates were also incubated with the other samples. Additionally, another negative control, to determine if the media used was sterile whereby the media was left overnight on the desk at 25°C. Incubation was set at 37ºC for the bacterial cultures for 24 hrs while the yeast was incubated at 37ºC for 48 hrs.

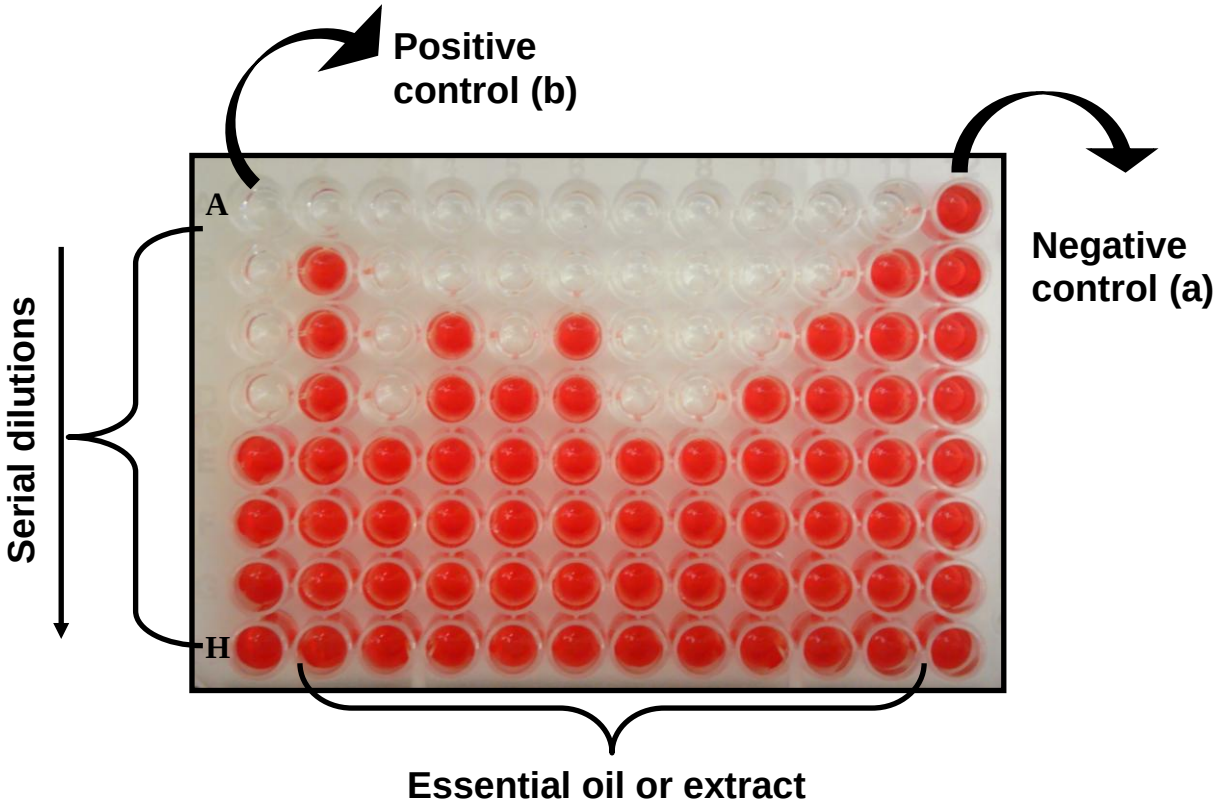


Figure 2.5 A typical micro-titre plate. (a) – negative control; (b) – positive control.

Microbial growth or inhibition was detected by using a 0.04% solution of INT (Sigma-Aldrich). An amount of 0.04 g was weighed and added to 100 ml sterile water to make up the required stock solution. INT is insoluble in cold water therefore for optimum solubility. It was placed in a water bath at approximately 55ºC for 30 min. Once dissolved, the INT solution was stored at 4°C and used within 2-3 days (Begue and Kline, 1972).

After relevant incubation periods, 40 µl of prepared INT solution was added to each of the 96 wells. For the bacteria, results were recorded six hrs later and for the yeast (*C. neoformans*) results were recorded 24 hrs later. All tests were performed in duplicate and on separate occasions to ensure accuracy and reproducibility of the results.

MIC values were interpreted using the classification adapted from Fabry et al. (1998) Aligiannis et al. (2001) and van Vuuren (2007 and 2008). Fabry et al. (1998) indicated that extracts having an MIC value of ≥8.0 mg/ml have some antimicrobial activity. Aligiannis et al. (2001), and Duarte et al. (2005), classified the interpretation of MIC values for plant materials, essential oils and extracts whereby strong inhibitors are those samples that have MIC values up to 0.5 mg/ml. Moderate inhibitors have MIC values between <0.5-1.5 mg/ml and weak inhibitors have MIC values above 1.6 mg/ml. Gibbons (2004) and Rios and Recio, (2005), as described in van Vuuren (2008), have interpreted the MIC value for any natural product below 1.0 mg/ml to have noteworthy antimicrobial activity and an MIC value below 0.1 mg/ml to be "very interesting". After extensive reviewing of the literature, van Vuuren (2008) proposed that essential oils with MIC values of 2.0 mg/ml or lower could be considered noteworthy. Thus incorporating all these interpretations, in this study, MIC values are interpreted as follows; an extract having an MIC value below 8.0 mg/ml will be considered to have some antimicrobial activity, but below 1 mg/ml is noteworthy. Any essential oil with an MIC of 2.0 mg/ml or lower will be considered as having noteworthy activity.

Furthermore, they can be divided into very strong inhibitors: MIC values less than 0.1 mg/ml; strong inhibitors: MIC values up to 0.5 mg/ml; moderate inhibitors: MIC values between 0.6-1.5 mg/ml; and weak inhibitors: MIC values above 1.6 mg/ml (Fabry et al., 1998; Aligiannis et al., 2001; Duarte et al., 2005; Rios and Recio, 2005; van Vuuren, 2008). A summary of the classification of antimicrobial activity used in this study is provided in Table 2.4.

2.4.3 Combination studies

In the testing of combinations of two or more plant essential oils or extracts, in order to assess whether synergy, antagonism or additive interactions are present, three general methods are used (Acar, 2000; Cassella et al., 2002). They are the chequerboard method, time-kill curves and disk diffusion methods (Acar, 2000). Results with killing curves and disk diffusion studies are qualitative assessments of interactions, while the micro-titre-plate method is expressed more quantitatively using the Fractional inhibitory concentration (FIC) method as well as the isobologram method (Acar, 2000). For the purposes of this study, combinations were tested using the micro-titre-plate method as it allows many different combinations and concentrations to be tested (Eliopoulos and Moellering, 1996; Acar, 2000).

Table 2.4 Classification of antimicrobial activity.

Type of sample	MIC value (mg/ml)	Classification
Extracts	<8.0	Some antimicrobial activity
	< 1.0	Noteworthy antimicrobial activity
Essential oils	≤2.0	Noteworthy antimicrobial activity
	>2.0 - <8.0	Some antimicrobial activity
Essential oils and extracts	<0.1	Very interesting antimicrobial activity/ Very strong inhibitors
	0.1 - 0.5	Strong inhibitors
	0.6 – 1.5	Moderate inhibitors

(Adapted from Fabry et al., 1998; Aligiannis et al., 2001; Gibbons, 2004; Duarte et al., 2005; Rios and Recio, 2005; van Vuuren, 2007 and 2008).

2.4.4 Fractional inhibitory concentration (FIC) determination

The ∑FIC is expressed as the interaction of two plant essential oils or extracts, wherein the concentration of each agent in combination is expressed as a fraction of the concentration that would produce the same effect when used independently (Berenbaum, 1978; van Vuuren and Viljoen, 2008). It is often used to analyze potential antimicrobial interactions. It does however, have certain limitations i.e. the MIC end points are required for each agents being tested (Stergiopoulou et al., 2008). If the MIC end point is not determined at the highest concentration tested, a ∑FIC cannot be calculated and thus for the purpose of this study it is

indicated as "not determined ([1])". However, results are interpreted by comparing the MIC values obtained for the plant essential oil or extracts alone and in combination. For all combinations where the MIC values were determined, the ∑FIC values were calculated according to Equation 2.2 and Equation 2.3 (Berenbaum, 1978; Davidson and Parish, 1989; O'Shaughnessy et al., 2006).

$$\mathrm{FIC_a} = \frac{\text{MIC of (a) in the combination with (b)}}{\text{MIC (a)independently}}$$

Equation 2.2

$$\mathrm{FIC_b} = \frac{\text{MIC of (b) in the combination with (a)}}{\text{MIC (b)independently}}$$

Equation 2.3

$_a$ and $_b$ represent the two plants. The sum of the FIC, known as the mean ∑FIC index was calculated using Equation 2.4

$$\sum \mathrm{FIC}_{\,\mathrm{index}} = \mathrm{FIC}_{\,a} + \mathrm{FIC}_{\,b}$$

Equation 2.4

The ∑FIC index has been adapted from Berenbaum (1978), Odds (2003) and modified to include an additive interpretation (Schelz et al., 2006; Iten et al., 2009; van Vuuren and Viljoen, 2011). It is used to determine the correlation between the two plants and may be classified as either synergistic (≤0.5), additive (0.5 - 1.0), indifferent/non-interactive (>1.0 - 4.0) or antagonistic (≥4.0). FIC determination was carried out on all the combinations associated with *A. afra*. This includes all the double and triple combinations of *A. afra* with other plants, as well as its combinations with adjuncts such as honey, salt, vinegar, brandy and milk. These combinations were tested and ∑FIC values determined against the pathogens used in this study i.e. *S. pneumoniae*, *S. pyogenes*, *S. agalactiae*, *M. smegmatis*, *E. faecalis*, *M. catarrhalis*, *K. pneumoniae* and *C. neoformans*, except where an endpoint MIC value was not noted. This was carried out as a preliminary determination of the presence of interactions within a combination for all pathogens. Thereafter four pathogens i.e. *K. pneumoniae*, *M. catarrhalis*, *E. faecalis* and *C. neoformans* were selected to further investigate interactions present, using isobologram construction studies.

Isobologram construction was undertaken, as it may be a more sensitive method to determine the *in vitro* pharmacodynamic interactions between combinations (Tallarida, 2006;

Stergiopoulou et al., 2008). An advantage over FIC determination is that a number of ratios can be tested and plotted out at the same time, in order to detect the interactions at different concentrations of the combination.

2.4.5 Isobologram construction

Isobolograms are used to characterize the nature of an interaction between combinations e.g. between plant essential oils or extracts, antibiotics or even between plants and antibiotics (Gennings et al., 1990). An isobol is a level curve, on the surface of an interaction among a combination of plant product extracts or essential oils. The surface is 3-dimensional, wherein the horizontal axes (x and y), represents the individual drug concentrations, and the vertical axes represent the effect of the combination. An isobol is thus the collection of all the points on the surface that lie at a particular effect (Boucher and Tam, 2006). An isobologram is a convenient graphical way of representing the results of a combination study and is useful in visualizing combination data (Tallarida and Raffa, 1995). These different types of interactions have been demonstrated and introduced by Fraser (1870-1871, 1872) and their approach has been extended by Loewe and Muischnek (1926), Loewe (1953) and Berenbaum (1981) (Gennings et al., 1990). More recently, they have been utilized in the combination study between antibiotics and commonly used essential oils by van Vuuren et al. (2009) and by Suliman et al. (2010) to determine the interactions between the essential oil of *A. afra* with other essential oils commonly used in traditional medicine in South Africa.

The isobologram ratio method (Berenbaum, 1978; Williamson, 2001) involves combining the two plants in nine ratios i.e. 9:1; 8:2; 7:3; 6:4; 5:5; 4:6; 3:7; 2:8; 1:9. The MIC was determined for all ratios and the plant samples independently. Micro-titre plates were then prepared as in Section 2.4.2. Ratios were calculated using Equations 2.5 and 2.6 and then plotted onto Microsoft Excel© as is demonstrated in Figure 2.6.

$$X = \frac{\text{MIC value of combination of essential oil/extract}}{\text{MIC value of essential oil/extract}}$$

Equation 2.5

$$Y = \frac{\text{MIC value of combination of essential oil/extract}}{\text{MIC value of essential oil/extract}}$$

Equation 2.6

MIC values (mg/ml) of plant samples in combination relative to the independent MIC were plotted on an isobologram, using GraphPad Prism® version 5 software, as a ratio, allowing for a graphical representation of the interaction of the various combinations.

Ratio values as calculated

100% test substance (plant X)

Well no	Concentrations (mg/ml)		MIC values obtained		Ratio values	
	Plant X	Plant Y	Plant X	Plant Y	Plant X	Plant Y
1	32	0	8.00	0.00	1.00	0.00
2	28.8	3.2	7.20	0.80	0.90	0.10
3	25.6	6.4	6.40	1.60	0.80	0.20
4	22.4	9.6	5.60	2.40	0.70	0.30
5	19.2	12.8	4.80	3.20	0.60	0.40
6	16	16	4.00	4.00	0.50	0.50
7	12.8	19.2	3.20	4.80	0.40	0.60
8	9.6	22.4	2.40	5.60	0.30	0.70
9	6.4	25.6	1.60	6.40	0.20	0.80
10	3.2	28.8	0.80	7.20	0.10	0.90
11	0	32	0.00	8.00	0.00	1.00

Ratios ranging from 9:1 to 1:9

100% test substance (plant Y)

Figure 2.6 Example of the ratios for essential oils and how they were presented in Microsoft Excel ©.

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 (≤0.5), additivity (>0.5 – 1.0), indifference (>1.0 – ≤4.0) and antagonism (>4.0) as shown in Figure 2.7. Tests were undertaken at least in duplicate. Conventional antimicrobials were included in all repetitions as positive controls. Negative controls were also included in the testing as described in Section 2.4.2.

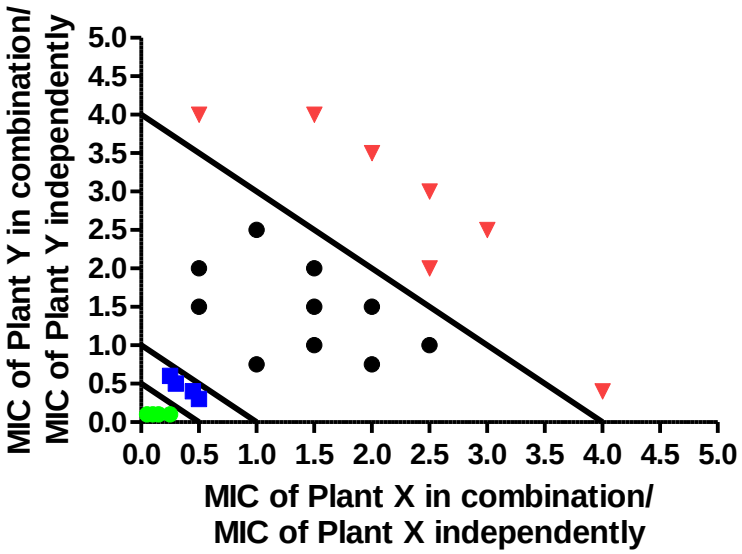


Figure 2.7 Example of an isobologram showing synergy (●), additivity (■), indifference (●) and antagonistic (▼) pharmacological interactions.

Chapter 3

The antimicrobial efficacy of *Artemisia afra* Jacq. ex Willd and *Lippia javanica* (Burm. f.) Spreng. in combination

3.1 Introduction

A. afra is combined with many different plants in South African traditional medicine, but most notably and consistently of all, with *Lippia javanica*. (Watt and Breyer-Brandwijk, 1962; Neuwinger, 2000; Huffman et al., 2002). A decoction of *L. javanica* is generally mixed with a decoction of wormwood and used for colds and influenza (Smith, 1895). Likewise, an infusion made with *A. afra* and *L. javanica*, is given to treat fevers, influenza (Hutchings et al., 1996), measles and used as a prophylactic against lung inflammations (Smith, 1895; Watt and Breyer-Brandwijk, 1962).

Although there has been much citation in literature regarding the use of the combination of these two plants, there is very little scientific evidence to support the traditional use in combination. Only one other study for the combination of *A. afra* and *L. javanica* has been researched by van Vuuren (2007). Time-kill assays were utilized to access the ethnobotanical use of *A. afra* essential oil (0.25%) alone, *L. javanica* essential oil (0.25%) alone and the 1:1 combination of *A. afra* (0.125%) with *L. javanica* (0.125%) essential oils. Time-kill on the two independent plants was carried out on three pathogens i.e. *K. pneumoniae*, *C. neoformans* and *B. cereus*. The time-kill using the combination was carried out against *K. pneumoniae*. Results on the combination study found that a bactericidal effect was achieved within an hour of exposure and that this cidal effect was maintained for the full 48 hrs of testing. The study concluded that *A. afra* interacts synergistically with *L. javanica* against *K. pneumoniae* thus validating the traditional use of the combination (van Vuuren, 2007)

The aims of this study included studying the essential oil composition of *A. afra* and *L. javanica* using GC-MS, TLC screening on the plants essential oils, dichloromethane: methanol extracts and the aqueous extracts independently and in combination (1:1) and testing of the antimicrobial activity, of *A. afra* and *L. javanica* independently as well as in combination using MIC microplate assays. Combinations were made by mixing different

ratios of *A. afra* with *L. javanica*. Nine ratios in total were carried out with either *A. afra* or *L. javanica* varying in mixed ratios. Additionally, not only the essential oils, but also the dichloromethane: methanol extracts as well as the aqueous extracts were tested. ∑FIC determination as well as isobologram studies were then done in order to determine the efficacy of *A. afra* with *L. javanica* in combination and identify any interaction between the two.

3.2 Results and discussion

3.2.1 Chromatographic techniques

3.2.1.1 Essential oil composition of *A. afra*

Thirty compounds were identified, making up 92.8% of the total composition (Table 3.1) in the essential oil of *A. afra*. The major compounds present were camphene (4.0%), 1, 8-cineole (17.0%), artemisia ketone (6.4%), β-thujone (6.0%), camphor (41.0%) and borneol (8.6%)

Table 3.1 GC-MS results of *A. afra*.

RRI*	Compounds	Area (%)
1016	α-Pinene	0.5
1057	**Camphene**	**4.0**
1104	β-Pinene	0.4
1193	α-Terpinene	0.1
1202	**1,8-Cineole**	**17.0**
1232	(Z)-β-Ocimene	0.4
1242	γ-Terpinene	0.1
1250	(E)-β-Ocimene	0.1
1281	Terpinolene	0.1
1350	**Artemisia ketone**	**6.4**
1441	**β-Thujone**	**6.0**
1488	α-Copaene	0.1
1501	Artemisia alcohol	0.2
1511	Chrysantenone	0.1
1521	**Camphor**	**41.0**
1573	Pinocarvone	0.5
1588	Bornyl acetate	0.5
1602	Terpinen-4-ol	1.7
1628	*cis-p*-Menth-2-en-1-ol	0.5
1639	Myrtenal	0.5
1653	*trans* Pinocarveol	1.0
1672	γ-Terpineol	0.2
1679	*trans* Piperitol	0.3
1701	α-Terpineol	0.3

RRI*	Compounds	Area (%)
1709	**Borneol**	**8.6**
1715	Germacrene D	0.9
1739	Piperitone	0.3
1741	Bicyclogermacrene	0.4
1745	Piperitol	0.3
1857	Myrtenol	0.3
Total		92.8

*RRI: Relative retention indices calculated against *n*-alkanes.
% calculated from TIC data.

Numerous studies have reported on the composition of the essential oil of *A. afra* (Appendix A). The sample of *A. afra* from this study is rich in camphor at 41.0% and β – thujone makes up only 6.0% of the total composition of the oil and α- thujone was absent. In the study by van Vuuren (2007), the essential oil of *A. afra* was collected from Klipriviersberg (the same location as this sample) and analysed. The major compounds detected in this study and that of van Vuuren (2007) were mostly the same except α-thujone (18.8%) was the major compound in van Vuuren (2007) and camphor (41.0%) was the major constituent in this study. As discussed in Section 3.4.2, the differences in the major compound composition of the oils are clearly noticed. This is not surprising, as studies conducted have concluded that *A. afra* shows immense chemical variation from individual plant-to-plant samples to collective plant samples (Graven et al., 1992; Chagonda et al., 1999; Mangena and Muyima, 1999; Viljoen et al., 2006b).

3.2.1.2 Essential oil composition of *L. javanica*

The GC-MS results for *L. javanica* showed twenty-one compounds (Table 3.2) making up 96.3%. The major compounds are 1, 8-cineole (2.3%), *p*-cymene (3.4%), *trans*-linalool oxide (4.5%), *cis*-linalool oxide (4.0%), linalool (70.7%) and caryophyllene oxide (6.9%).

Viljoen et al. (2005) as well as van Vuuren (2007) studied the chemical composition of *L. javanica* essential oil from Fairlands in the North of Johannesburg, the same locality as the *L. javanica* used in this study. Adequate comparison of the differences in the major compounds can thus be made. In Viljoen et al. (2005), linalool (65.19%) was found to be the major compound followed by (*Z*)-β-ocimene (12.97%) and (*E*)-β-ocimene (6.21%). van Vuuren (2007) found the major compounds to be (*Z*)-β-ocimene (13.0%) and linalool (65.2%). Comparing these samples with that of *L. javanica* essential oil in this study, it is clear that

linalool is the main or dominant compound, with the composition of linalool alone in all three samples greater than 65.0%. A slight difference that is noted is the absence of (*Z*)-β-ocimene in the sample tested in this study. In van Vuuren (2007), (*Z*)-β-ocimene was only found as a major constituent in the Fairland population and was not a main constituent in the other populations tested. Thus, the absence of this constituent, in the sample used in this study, is notable although variations may exist between and within natural populations (Viljoen et al., 2005).

Table 3.2 GC-MS results of *L. javanica*.

RRI*	**Compounds**	**Area (%)**
1016	α-Pinene	0.1
1057	Camphene	0.2
1117	Sabinene	0.1
1193	Limonene	0.5
1202	**1,8-Cineole**	**2.3**
1270	***p*-Cymene**	**3.4**
1441	***trans*-Linalool oxide**	**4.5**
1464	α-Longipinene	1.2
1471	***cis*-Linalool oxide**	**4.0**
1488	α-Copaene	0.3
1511	Chrysanthenone	0.1
1521	Camphor	0.4
1546	**Linalool**	**70.7**
1575	d-Terpineol	0.1
1596	β-Caryophyllene	0.3
1602	Terpinen-4-ol	0.2
1701	α-Terpineol	0.1
1709	Borneol	0.8
1728	α-Amorphene + α-muurolene	0.1
2010	**Caryophyllene oxide**	**6.9**
Total		96.3

*RRI: Relative retention indices calculated against *n*-alkanes
% calculated from TIC data

3.2.1.3 Thin layer chromatography

TLC fingerprint studies were carried out as an initial screening process singularly and in combination, in order to determine and visualise the locality of the various compounds by using R_f values. Figure 3.1, 3.2 and 3.3 show respectively the TLC results for essential oils, dichloromethane: methanol extracts and aqueous extracts of *A. afra*, *L. javanica* alone and in combination.

In Figure 3.1, compounds not visible under UV light of 254 nm or UV of 365 nm or white light are then seen when the TLC plate has been derivatized. Several compounds in *A. afra* essential oil were detected under 254 nm of UV light.

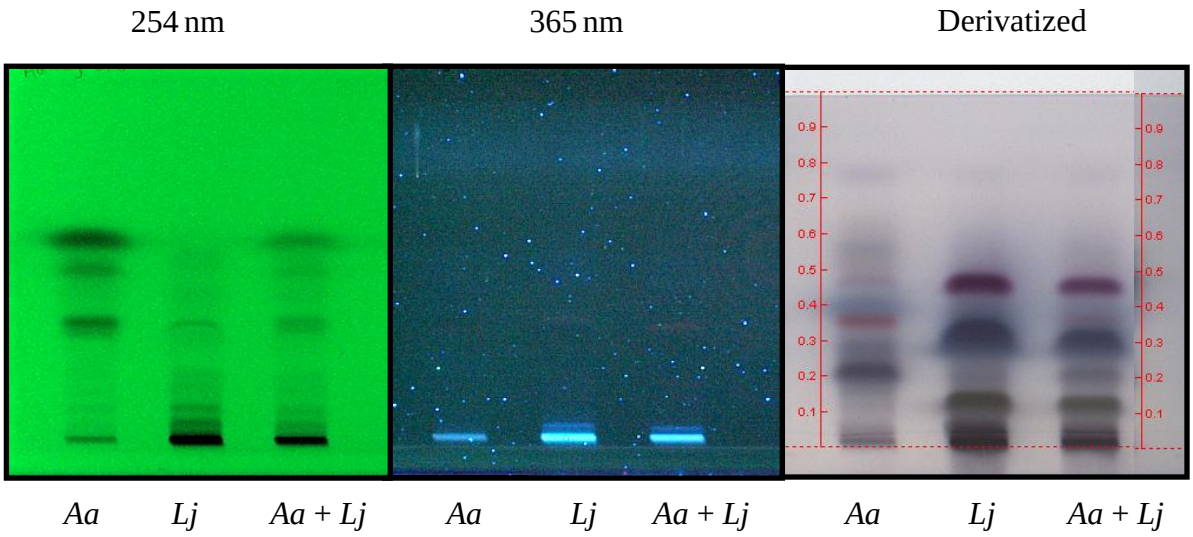


Figure 3.1 TLC of the essential oils of *A. afra*, *L. javanica* individually and in combination under UV light of 254 nm, UV light of 365 nm and visualized with vanillin-sulphuric acid reagent. *Aa - A. afra*; *Lj - L. javanica*; *Aa + Lj -A. afra* with *L. javanica*.

The combination of *A. afra* and *L. javanica* essential oil showed a mixture of the components of each of the oils, although to a lesser degree. Under UV light of 365 nm, compounds at the starting point for all three lanes were visible. A single compound, red in colour with R_f = 0.35, was visible in *L javanica* alone, as well as in the combination with *A. afra*. After derivatization with vanillin-sulphuric acid reagent, many compounds not previously visible were observed.

The dichloromethane: methanol extracts (Figure 3.2) showed good separation of the constituents of each plant under UV light of 254 nm. The combination of *A. afra* with *L. javanica* also showed very clearly the compounds that are visible singularly. The derivatized plate also demonstrated good separation with a greenish-yellow coloured major compound found in *L. javanica* (R_f = 0.45). This compound was also clearly visible in the combination wherein *A. afra* was combined with *L. javanica*.

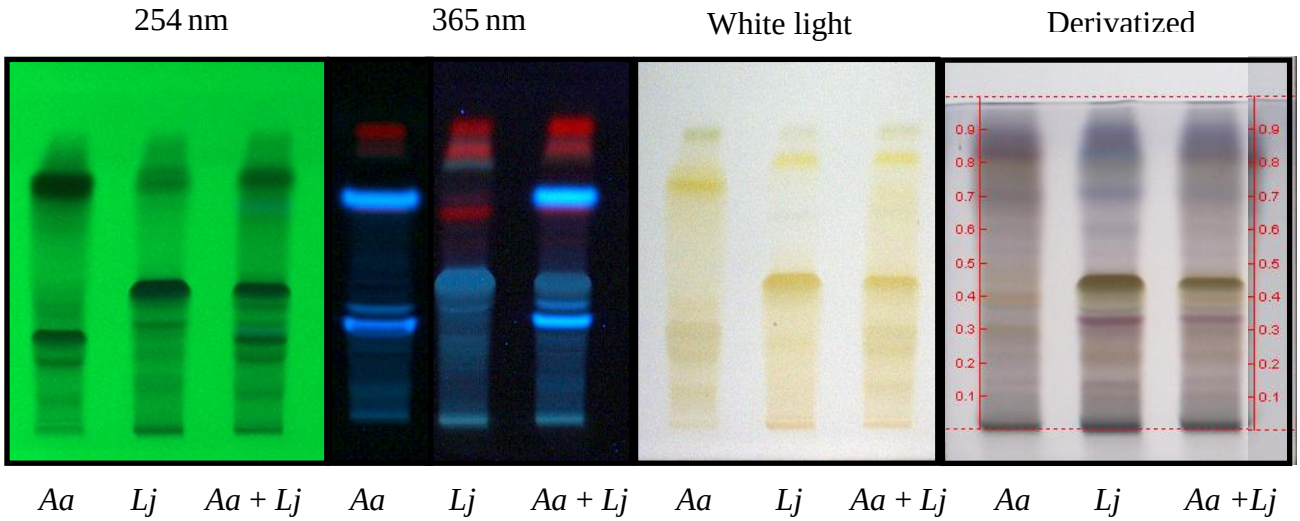


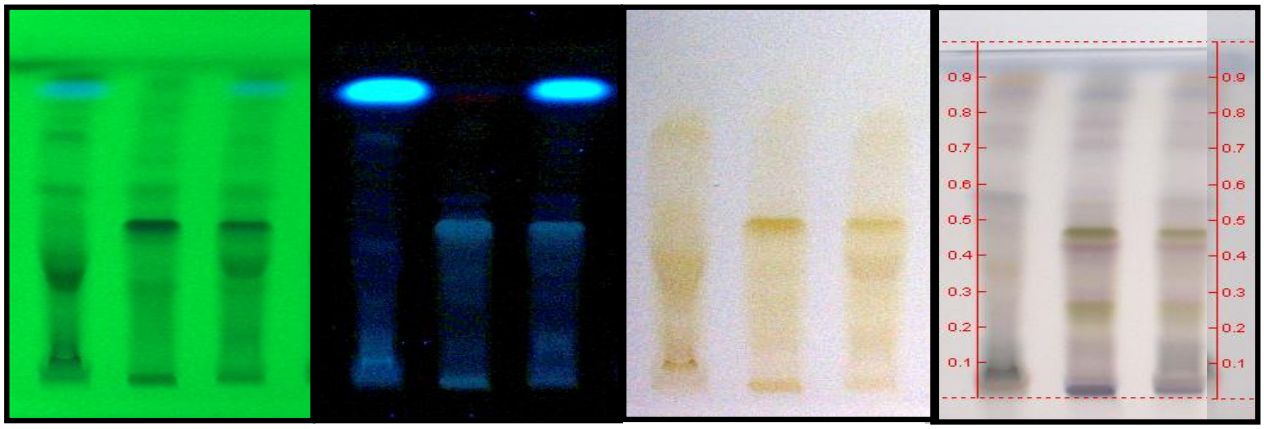
Figure 3.2 TLC of the dichloromethane: methanol extracts of *A. afra*, *L. javanica* individually and in combination under UV light of 254 nm, under UV light of 365 nm, visualized under white light and visualized with vanillin-sulphuric acid reagent. *Aa* - *A. afra*; *Lj* - *L. javanica*; *Aa* + *Lj* -*A. afra* with *L. javanica*.

The TLC chromatograms of the aqueous extracts (Figure 3.3) of *A. afra*, *L. javanica* and their combination showed many compounds at 254 nm and 365 nm. After derivatization, compounds in *L. javanica* that were present in the dichloromethane: methanol extract were noted in the aqueous extracts of *L. javanica* and in the combination with *A. afra* at $R_f = 0.45$.

The HPTLC chromatograms show the complexity of the different plants. This is increased when the plants are combined. Thus, the individual components of the plants singularly and in combination with other plants would be very difficult to identify.

The presence of a greater number of bands in the combination TLC shows that combining plants together creates complex phytochemical pools and therefore more possibilities for synergistic interactions between the compounds that are now in contact with each other. There are also increased opportunities for antimicrobial interaction against bacteria as different compounds may inhibit microbes using different mechanisms thus resulting in synergy.

254 nm 365 nm White light Derivatized



Aa *Lj* *Aa + Lj* *Aa* *Lj* *Aa + Lj* *Aa* *Lj* *Aa + Lj* *Aa* *Lj* *Aa +Lj*

Figure 3.3 TLC of the aqueous extracts of *A. afra*, *L. javanica* individually and in combination under UV light of 254 nm, under UV light of 365 nm, visualized under white light and visualized with vanillin-sulphuric acid reagent. *Aa - A. afra*; *Lj - L. javanica*; *Aa + Lj -A. afra* with *L. javanica*.

3.2.2 Antimicrobial analysis

3.2.2.1 MIC assays and FIC determination

MIC values obtained for the negative controls, ciprofloxacin and amphotericin B, against the micro-organisms used in the study are shown in Table 3.3. MIC values were interpreted and classified according to Table 2.4, Chapter 2. The negative control, acetone, was noted immediately when checking the results of the experiment and where problems were experienced tests were repeated.

Table 3.3 MIC values obtained for controls (µg/ml)

Micro-organism	**Controls**
	Ciprofloxacin/Amphotericin B
M. catarrhalis ATCC 23246	6.3
K. pneumoniae NCTC 9633	0.2
E. faecalis ATCC 29212	0.6
C. neoformans ATCC 90112	3.1
S. pneumoniae ATCC 49619	0.3
S. pyogenes ATCC 8668	0.6
S. agalactiae ATCC 55618	1.3
M. smegmatis (clinical)	0.2

Culture purity and signs of contamination were checked by streaking out a loopful of culture onto agar and incubating as necessary and checked immediately after completion of incubation. Where antimicrobial controls did not reflect consistent expected ranges the entire experiment was repeated and the result deleted from the data set. Generally, the MIC values for *A. afra* and *L. javanica* were in the moderate range and showed good consistency from sample to sample.

3.2.2.1.1 Essential oil

A. afra has a broad spectrum of activity (Mangena and Muyima 1999) The MIC results of the essential oils of *A. afra* (Table 3.4) showed poor antimicrobial activity against *K. pneumoniae, M. catarrhalis* and *E. faecalis* with mean MIC values at 8.0, 8.7 and 8.7 mg/ml respectively. Some antimicrobial activity was seen against *C. neoformans* (3.8 mg/ml), *S. pneumoniae* (3.3 mg/ml) and *S. agalactiae* (4.8 mg/ml).

Table 3.4 MIC and ∑FIC values of *A. afra, L. javanica* essential oils alone and in combination against different pathogens (mg/ml).

Micro-organism	**MIC (mg/ml)**			**∑FIC**	
	A. afra	***L. javanica***	***A. afra* with *L. javanica***	***A. afra* with *L. javanica* (1:1)**	**Interpretation**
K. pneumoniae NCTC 9633	8.0	8.0	8.0	1.0	Additive
M. catarrhalis ATCC 23246	8.7	8.0	8.0	1.0	Additive
E. faecalis ATCC 29212	8.7	8.0	8.0	1.0	Additive
C. neoformans ATCC 90112	3.8	**1.5**	**2.0**	0.9	Additive
S. pneumoniae ATCC 49619	3.3	**2.0**	**2.0**	0.8	Additive
S. pyogenes ATCC 8668	**1.7**	**1.4**	**1.0**	0.7	Additive
S. agalactiae ATCC 55618	4.8	4.0	4.0	0.9	Additive
M. smegmatis (clinical)	**1.5**	**2.0**	**2.0**	1.2	Indifferent

* Values in bold indicate noteworthy activity.
Controls excluded for the sake of brevity. Please refer to Chapter 3 for further detail regarding control values.

Noteworthy antimicrobial activity was seen against *S. pyogenes* and *M. smegmatis* whereby mean MIC values of 1.7 and 1.5 mg/ml were obtained. Thus *A. afra* essential oil was the

most active in inhibiting the growth of *M. smegmatis* and can be considered a moderate antimicrobial inhibitor.

The antimicrobial activity of *A. afra* essential oil has been widely studied using the disc diffusion method or the MIC micro-titre plate method. In order to further substantiate the results obtained by disk diffusion and the MIC assays, time-kill studies were employed. In a disc diffusion study by Graven et al. (1992), *A. afra* essential oil was found to be active against *Acinetobacter calcoaceticus*, *Beneckea natriegens*, *Brevibacterium linens*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *E. faecalis*, *Moraxella* spp. and *Serratia marcescens*.

A. afra also exhibits known fungicidal activity as described in the study by Gundidza (1993). It was shown that the essential oil has a strong inhibitory effect on *Candida albicans*, *Aspergillus niger*, *Alternia alternate*, *Aspergillus ochraceus*, *Geotrichum candidum*, *Penicillium citrium* and *Aspergillus parasiticus*. A later study by Mangena and Muyima (1999) using the disc diffusion assay on the essential oil of *A. afra* indicated that it had a broad spectrum antimicrobial activity. Among the most sensitive pathogens to the oil were *S. pyogenes*, *Listeria monocytogenes* and *Acinetobacter johnsonii*. *S. pyogenes* was used in this study as well and noteworthy antimicrobial activity was also seen with *A. afra* essential oil with a mean MIC value of 1.7 mg/ml. Due to the different methods used to assess the antimicrobial activity, direct comparisons cannot be made. However, the antimicrobial activity noted in this study and the one by Mangena and Muyima (1999) serves to confirm the inhibitory effect of *A. afra* on *S. pyogenes* which is a common cause of respiratory related conditions (Khan et al., 2009b). In 2002, Muyima et al. conducted a study on the antimicrobial activity of *A. afra* essential oil. Results found using the disc diffusion assay showed a high level of sensitivity of *A. afra* towards *E. coli*, *S. aureus*, *Candida albicans* and *Ralstonia pickettii*.

Huffman et al. (2002) examined the antimicrobial activity of the essential oil of *A. afra* using the microplate method and minimum inhibitory percentages were determined. *Candida albicans* and *C. neoformans* gave minimum inhibitory percentages of 0.25%. In 2006, van Vuuren and Viljoen conducted a study on the antimicrobial activity of the essential oil of *A. afra* using the MIC micro-titre plate and the time-kill method. The essential oil was tested against ten pathogens including *S. aureus*, *S. epidermidis*, *B. cereus*, *B. subtilis*, *E. coli*, *P.*

aeruginosa, *E. faecalis*, *K. pneumoniae*, *C. albicans* and *C. neoformans*. *A. afra* exhibited MIC values of 5.7, 10.1 and 8.0 mg/ml against *E. faecalis*, *K. pneumoniae* and *C. neoformans*, respectively. These results are in congruence with those attained in this current study. Time-kill studies were performed against *S. aureus*, *K. pneumoniae* and *C. albicans*. The time-kill study performed against *S. aureus* and *C. albicans* revealed bactericidal efficacy after four hours of exposure to the essential oil of *A. afra* (0.5%). However, against *K. pneumoniae*, regrowth was noted after one hour of exposure.

In another time-kill study by Viljoen et al. (2006b) carried out on *A. afra* essential oil, *A. afra* was tested against *K. pneumoniae* and *C. neoformans* over time to determine if a bactericidal effect was apparent in order to validate the traditional use for respiratory conditions. Against *K. pneumoniae* a bactericidal effect was noted at 0.75% within 10 min of the test. A bactericidal effect was noted for 1.0% of *A. afra* against *C. neoformans* within 60 min. Thus, the traditional use of *A. afra* essential oil was validated for the treatment of respiratory conditions.

It is important to note that *A. afra* essential oil, to my knowledge, has never been tested before using the MIC micro-titre plate method against *S. pyogenes*, *S. agalactiae* and *S. pneumoniae*. This is somewhat surprising as these are key pathogens in the causes of respiratory conditions in South Africa and in the world. Furthermore, activities found for *S. pyogenes* were one of the best of all pathogens tested.

L. javanica essential oil showed poor antimicrobial activity against *K. pneumoniae, M. catarrhalis* and *E. faecalis* with mean MIC values of 8.0 mg/ml. Against *S. agalactiae*, some antimicrobial activity was noted with a mean MIC value of 4.0 mg/ml. Against *S. pneumoniae* and *M. smegmatis*, noteworthy activity was seen, both giving a mean MIC value of 2.0 mg/ml. The best antimicrobial activity of *L. javanica* essential oil was seen against *C. neoformans* and *S. pyogenes* that gave mean MIC values of 1.5 and 1.4 mg/ml respectively. *L. javanica* essential oil can be further classified as a moderate inhibitor of *C. neoformans* and *S. pyogenes.* The noteworthy antimicrobial activity seen against *C. neoformans, S. pneumoniae*, *S. pyogenes* and *M. smegmatis* therefore supports the traditional use of *L. javanica*.

Antimicrobial efficacies for *L. javanica* essential oil has been demonstrated against *E. coli*, *S. aureus*, *Salmonella gallnarum*, *Klebsiella pneumoniae*, *Candida albicans* *P. aeruginosa*, *S. epidermidis*, *E. cloacae* (Mwangi et al., 1992; Chagonda et al., 1993; Ngassapa et al., 2003; Subramoney, 2003; Manenzhe et al., 2004).

Samie et al. (2005) conducted a study testing the essential oil of *L. javanica* using the disc diffusions assay as well as the micro-dilution method. Disk diffusion showed activity against *B. pumilus*, *B. subtilis*, *E. faecalis*, *E. cloacae*, *E. coli*, *P. mirabilis* and *K. pneumoniae*. The micro-dilution method revealed activity against *B. cereus*, *B. pumilus*, *B. subtilis*, *S. aureus*, *E. faecalis*, *E. cloacae*, *E. coli*, *P. agglomerans*, *P. aeruginosa*, *S. flexneri*, *A. hydrophila P. mirabilis*, *K. pneumoniae* and *S. cholera-suis*. The MIC values obtained by Samie et al. (2005) against *E. faecalis* and *K. pneumoniae* were 3.0 mg/ml. These were lower than that noted in this current study where MIC values of 8.0 mg/ml were noted. Different geographical locations for collection of *L. javanica* could be the reason behind the differences in MIC values. It is known that *L. javanica* essential oil exhibits chemical variation with respect to geographical distribution (Viljoen et al., 2005) and this may contribute to the varied antimicrobial effects noted.

Time-kill studies by Viljoen et al. (2005) against *K. pneumoniae* showed bactericidal effect within 30 min for all the concentrations tested. Against *C. neoformans*, the killing rate at 1% concentration was within one hour for *L. javanica.* No bactericidal effect was noted against *B. cereus* over a 24 hr period. These results validated the use of *L. javanica* in African traditional healing to treat the symptoms of colds and flu (Viljoen et al., 2005).

van Vuuren and Viljoen (2006) tested the antimicrobial activity of *L. javanica* essential oil from the same locality against *S. aureus*, *S. epidermidis*, *B. cereus*, *B. subtilis*, *E. coli*, *P. aeruginosa*, *E. faecalis*, *K. pneumoniae*, *C. albicans* and *C. neoformans*. MIC values obtained against *E. faecalis* were 13.5 mg/ml, *K. pneumoniae*, 7.6 mg/ml and *C. neoformans*, 2.4 mg/ml. Congruency between results of the present study was noted. A time-kill study was also performed by van Vuuren and Viljoen (2006) using 0.5% essential oil of *L. javanica* against *S. aureus*, *K. pneumoniae* and *C. albicans*. Against *S. aureus* a 50% reduction in CFU/ml was noted over 24 hrs however, no bactericidal activity was noted against *K. pneumoniae*. Against *C. albicans*, bactericidal activity was only noted after 24 hrs of exposure. These differences in activities seen between Viljoen et al. (2005), and van Vuuren

and Viljoen (2006), wherein the samples of *L. javanica* was from the same locality, are possibly due to the qualitative and quantitative variation seen between and within the populations of *L. javanica*. These variations occur where even a minor compound could affect the antimicrobial activity of the plant essential oil. This demonstrates that the possibilities of synergistic, antagonistic or even additive interactions are not to be ignored (Nakatsu et al., 2000; van Vuuren and Viljoen, 2006).

The combination of *A. afra* with *L. javanica* essential oils showed poor antimicrobial activity against *K. pneumoniae, M. catarrhalis* and *E. faecalis* giving mean MIC values of 8.0 mg/ml. No increase in the MIC value, as compared to the individual oils, was noted. Some antimicrobial activity was seen against *S. agalactiae* with a mean MIC value of 4.0 mg/ml. Noteworthy antimicrobial activity was noted against *C. neoformans, S. pneumoniae* and *M. smegmatis* with mean MIC values of 2.0 mg/ml. However, against *S. pyogenes* a mean MIC value of 1.0 mg/ml was noted. The combination is thus classified as a moderate inhibitor of *S. pyogenes*. This was the most sensitive pathogen to the combination of the essential oils of *A. afra* with *L. javanica*.

∑FIC values calculated from the combination of *A. afra* with *L. javanica* essential oil against *K. pneumoniae, M. catarrhalis, E. faecalis, C. neoformans, S. pneumoniae, S. pyogenes* and *S. agalactiae* ranged from 0.7-1.0. This indicates that the combination showed additive interactions against these pathogens. Only one pathogen i.e. *M. smegmatis* showed indifferent interactions for the combination with a ∑FIC value of 1.2. It is important to note that no antagonism against the pathogens tested was noted for the combination.

The combination of *A. afra* with *L. javanica* essential oils has been tested against one pathogen using a time kill assay (van Vuuren, 2007), as mentioned in Section 3.1. There have been numerous citations on the use of the combination in traditional African medicine (Smith, 1895; Watt and Breyer-Brandwijk, 1962; Hutchings et al., 1996; Neuwinger, 2000; Huffman et al., 2002). *L. javanica* is cited to be used in combination with *Aloysia triphylla* as an herbal tea for the treatment of colds, fever and asthma (van Wyk and Wink. 2004).

However, scientific validation of the efficacy of the combination is nowhere to be found. Even though van Vuuren (2007) has studied the combination, different methodologies were not used in order to assess the presence of various interactions and against different micro-

organisms. Since no previous studies on the combination have been done, comparisons cannot be made. However, it should be noted that no antagonism was seen in the combination against any of the pathogens tested. Mostly additive interactions were prominent and where additivity was absent, indifference was evident. Further interactive efficacies are elaborated on in Section 3.2.2.2.

3.2.2.1.2 Dichloromethane: methanol extracts

The MIC values observed for the dichloromethane: methanol extracts, as shown in Table 3.5, of *A. afra* showed some antimicrobial activity against *K. pneumoniae* (6.3 mg/ml), *M. catarrhalis* (4.7 mg/ml), *E. faecalis* (6.3 mg/ml), *C. neoformans* (1.2 mg/ml), *S. pyogenes* (1.1 mg/ml), *S. agalactiae* (3.0 mg/ml) and *M. smegmatis* (1.7 mg/ml). The antimicrobial activity of *A. afra* against *C. neoformans* and *S. pyogenes* (MIC values of 1.2 and 1.1 mg/ml) can further classify *A. afra* dichloromethane: methanol extract as a moderate inhibitor of these two pathogens. Noteworthy and probably the best antimicrobial activity were seen for *A. afra* against *S. pneumoniae* where a mean MIC value of 0.5 mg/ml was obtained. *A. afra* dichloromethane: methanol extract can thus be further classified as being a strong inhibitor of *S. pneumoniae*.

A. afra methanol extracts were tested for antimicrobial activity using the disc diffusion assay by Rabe and van Staden (1997) and activity was noted against *S. aureus* and *B. subtilis*. McGaw et al. (2000) later performed disc diffusion assays as well as MIC dilution assays to determine the antimicrobial activity of *A. afra*. The hexane extract and the ethanol extracts were tested. No activity against the pathogens at the concentration tested (12.5 mg/ml) was seen with the hexane extracts. The ethanol extracts however, showed activity against *B. subtilis* and *S. aureus* when using both antimicrobial assays. Braithwaite et al. (2008) studied the methanol and the acetone extracts of *A. afra* to validate smoke inhalation therapy to treat some microbial infections. The MIC micro-titre plate assay was used and noteworthy activity (<1.0 mg/ml) was seen only for the acetone extracts against *S. aureus* (MIC value = 0.25 mg/ml), *B. cereus* (MIC value = 0.25 mg/ml) and *C. neoformans* (MIC value = 0.75 mg/ml). Some antimicrobial activity was noted with the acetone extract of *A. afra* against *K. pneumoniae* with an MIC value of 2.0 mg/ml. Results obtained in this study vary slightly compared to previous studies mentioned. However, direct comparisons are difficult as the type of extracts and the pathogens tested are different.

Table 3.5 MIC and ∑FIC values of *A. afra* and *L. javanica* dichloromethane: methanol extracts alone and in combination against different pathogens (mg/ml).

Micro-organism	MIC (mg/ml)			∑FIC	
	A. afra	***L. javanica***	***A. afra* with *L. javanica***	***A. afra* with *L. javanica* (1:1)**	**Interpretation**
K. pneumoniae NCTC 9633	6.3	16.0	6.0	0.7	Additive
M. catarrhalis ATCC 23246	4.7	6.0	6.0	1.1	Indifferent
E. faecalis ATCC 29212	6.3	2.5	4.7	1.3	Indifferent
C. neoformans ATCC 90112	1.2	3.5	2.0	1.1	Indifferent
S. pneumoniae ATCC 49619	**0.5**	**0.4**	**0.6**	1.4	Indifferent
S. pyogenes ATCC 8668	1.1	**0.4**	1.5	2.6	Indifferent
S. agalactiae ATCC 55618	3.0	2.2	1.1	**0.4**	**Synergy**
M. smegmatis (clinical)	1.7	1.5	2.5	1.6	Indifferent

* Values in bold indicate noteworthy activity.
Controls excluded for the sake of brevity. Please refer to Chapter 3 for further detail regarding control values.

When Mativandlela et al. (2008) tested the ethanol extracts of *A. afra* against *M. smegmatis*; an MIC value of 1.56 mg/ml was obtained. This result is in congruence with the findings in this study where the mean MIC value obtained against *M. smegmatis* was 1.7 mg/ml. This report by Mativandlela et al. (2008) together with the results from this study provides evidence to support the use of *A. afra* against certain respiratory pathogens.

L. javanica dichloromethane: methanol extract displayed very poor antimicrobial activity against *K. pneumoniae* with a mean MIC value of 16.0 mg/ml. MIC values ranging from 2.2 - 6.0 mg/ml, showing some antimicrobial activity, were obtained against *S. agalactiae, E. faecalis, C. neoformans* and *M. catarrhalis*. Moderate inhibition was obtained against *M. smegmatis* with a MIC value of 1.5 mg/ml. Noteworthy activity with strong inhibition was noted for *L. javanica* against *S. pneumoniae* and *S. pyogenes* with mean MIC values of 0.4 mg/ml.

L. javanica methanol, acetone and hexane extracts have been tested for their antimicrobial activity (Samie et al., 2005) using the disc diffusion and the MIC micro-titre plate method. The methanol extract of the leaves were active against *B. cereus, B. subtilis, E. faecalis, E. cloacae, E. coli, S. flexneri, A. hydrophila* and *S. cholera-suis* with MIC values ranging between 1.5-6.0 mg/ml. Against *K. pneumoniae* poor activity was noted with an MIC value of 12.0 mg/ml. Against *E. faecalis*, some antimicrobial activity was evident where a MIC value of 3.0 mg/ml was noted. This correlates with the results found in this study wherein MIC values against *K. pneumoniae* and *E. faecalis* were 16.0 and 2.5 mg/ml respectively. The acetone extracts exhibited similar activities to the methanol extracts with MIC values of 6.0 mg/ml against *E. faecalis* and 12.0 mg/ml against *K. pneumoniae*. The hexane extracts exhibited less activity against the tested pathogens with poor antimicrobial activity against *E. faecalis* and *K. pneumoniae* (12.0 mg/ml).

The methanol extracts of *L. javanica* were similarly tested against four pathogens i.e. *S. aureus, E. faecalis, E. coli* and *P. aeruginosa* (Shikanga et al., 2010). Noteworthy activities were noted with all the MIC values below 1.0 mg/ml. Relevant to the current study, was the MIC value obtained against *E. faecalis* of 0.14 mg/ml. This denoted very strong inhibition and very interesting activity. However, this was not a result that was demonstrated in the current study where the MIC value obtained was 2.5 mg/ml. This difference could be due to the differing collection localities and/or the chemical variations between and in natural populations (Viljoen et al., 2005).

Samie at el. (2010) tested the acetone and the hexane extracts of the leaves of *L. javanica* against three fungal organisms. Antifungal activity was noted for the acetone extracts against *Candida krusei* (MIC value of 1.88 mg/ml). The hexane extracts showed activity against *C. albicans* and *C. krusei*, both with MIC values of 3.75 mg/ml. No activity against *C. neoformans* (MIC value of >7.5 mg/ml) at the highest concentration tested was noted for either the acetone or the hexane extracts. However, in the current study some activity (MIC value = 3.5 mg/ml) was noted against *C. neoformans*. Again, geographical and chemical variations may be a contributing factor.

L. javanica dichloromethane: methanol extracts have not previously been tested against *S. pneumoniae* and *S. pyogenes* (where best activities were noted in this study). This is

surprising as *Streptococcus* spp are well known respiratory organisms and the traditional use of *L. javanica* is for the treatment of just such infections. The results obtained in this study, however, serve to validate the use of *L. javanica* in the treatment of *S. pneumoniae* (bacterial pneumoniae) and *S. pyogenes* ("strep" throat) related infections.

The combination of *A. afra* with *L. javanica* dichloromethane: methanol extracts showed some antimicrobial activity against *K. pneumoniae*, *M. catarrhalis*, *E. faecalis*, *C. neoformans* and *M. smegmatis* with MIC values ranging from 2.0-6.0 mg/ml. Moderate inhibition was noted against *S. pyogenes* and *S. agalactiae*. The best antimicrobial activity with noteworthy inhibition was found against *S. pneumoniae* with an MIC value of 0.6 mg/ml.

∑FIC values calculated showed indifferent interactions for all the pathogens tested with the exception of *K. pneumoniae* and *S. agalactiae*. An additive interaction was noted against *K. pneumoniae* with a ∑FIC value of 0.7. A synergistic interaction of the combination of *A. afra* with *L. javanica* was seen with ∑FIC = 0.4 against *S. agalactiae*.

The particular combination of *A. afra* with *L. javanica* dichloromethane: methanol extracts have also not been studied previously. From this study, the ∑FIC results did not show any antagonistic interactions. In fact, additivity was noted against *K. pneumoniae* (∑FIC value of 0.7). Synergy was observed for *S. agalactiae* (∑FIC value of 0.4). As *K. pneumoniae* is a leading cause of nosocomial pneumoniae (Ko et al., 2002; Yu et al., 2007; Brisse et al., 2009) in South Africa; this may prove to be a useful discovery.

3.2.2.1.3 Aqueous extracts

The aqueous extracts of *A. afra* (Table 3.6) showed generally poor antimicrobial activity against the pathogens tested with some antimicrobial activity against *C. neoformans* (4.0 mg/ml) and *S. pyogenes* (5.3 mg/ml). The best activity of *A. afra* aqueous extract was seen against *S. agalactiae* giving weak antimicrobial activity with a mean MIC value of 1.7 mg/ml. poor antimicrobial activity was noted against *M. catarrhalis*, *S. pneumoniae* and *M. smegmatis*, all yielding mean MIC values of 8.0 mg/ml while against *E. faecalis* a MIC value of 7.0 mg/ml was obtained. Very poor activity was seen against *K. pneumoniae* where a mean MIC value of 12.0 mg/ml was noted.

Table 3.6 MIC and ∑FIC values of *A. afra*, *L. javanica* aqueous extracts alone and in combination against different pathogens (mg/ml).

Micro-organism	MIC (mg/ml)			∑FIC	
	A. afra	***L. javanica***	***A. afra* with *L. javanica***	***A. afra* with *L. javanica* (1:1)**	**Interpretation**
K. pneumoniae NCTC 9633	12.0	≥16.0	12.0	ND[1]	Additive[2]
M. catarrhalis ATCC 23246	8.0	≥16.0	8.0	ND[1]	Additive[2]
E. faecalis ATCC 29212	7.0	12.0	8.0	0.9	Additive
C. neoformans ATCC 90112	4.0	3.5	3.0	0.8	Additive
S. pneumoniae ATCC 49619	8.0	4.0	8.0	1.5	Indifferent
S. pyogenes ATCC 8668	5.3	3.0	4.0	1.0	Additive
S. agalactiae ATCC 55618	1.7	**0.5**	**0.8**	1.0	Additive
M. smegmatis (clinical)	8.0	6.0	8.0	1.2	Indifferent

* Values in bold indicate noteworthy activity.
ND[1] No ∑FIC value could be calculated as no MIC end point for *L. javanica* was obtained at the highest concentration tested.
[2] Tentative interpretation according to MIC data.
Controls excluded for the sake of brevity. Please refer to Chapter 3 for further detail regarding control values.

The aqueous extracts of *A. afra* were tested by Rabe and van Staden (1997) and no activity at the concentration tested (100.0 mg/ml) was noted when using the disc diffusion method for antimicrobial assessment. McGaw et al. (2000) also tested *A. afra* aqueous extracts and found no antimicrobial activity using the disc diffusion method as well as the MIC micro-titre plate method. Aqueous extracts have been known to exhibit poor to no antimicrobial activity (Jäger, 2003; Abu-Shanab et al., 2004; van Vuuren, 2008). This is in contrast to the traditional method of preparation of plants where most often the infusion or the decoctions are used.

L. javanica aqueous extract showed some antimicrobial activity against *C. neoformans*, *S. pneumoniae*, *S. pyogenes* and *M. smegmatis* with mean MIC values ranging between 3.0-6.0 mg/ml. Against *K. pneumoniae* and *M. catarrhalis*, *L. javanica* was not active at the highest

concentration tested while very poor activity was seen against *E. faecalis* with a mean MIC value of 12.0 mg/ml. Interestingly, noteworthy strong antimicrobial inhibition was noted against *S. agalactiae* where a mean MIC value of 0.5 mg/ml was obtained.

Thembo et al. (2010) tested the aqueous extracts of *L. javanica* and found no activity against some mycotoxigenic fungi. However, results from the current study provided an exception against *S. agalactiae* wherein strong inhibitory activity (MIC value of 0.5 mg/ml) was noted. *S. agalactiae* is a respiratory tract pathogen and causes pneumonia in healthy humans. The use of *L. javanica* infusions and decoctions in traditional medicine may have solid scientific support due to the inhibition observed in this study. Shikanga et al. (2010) tested the methanol extracts of *L. javanica* as a substitute of the aqueous extracts as the extract compositions were similar and the methanol extracts are easier to dry. The study was performed to determine the antioxidant and antibacterial activity of *L. javanica* infusions. Results were promising with noteworthy activities recorded.

When *A. afra* was combined with *L. javanica* aqueous extract very poor antimicrobial activity was noted against the pathogen *K. pneumoniae* (MIC value of 12.0 mg/ml) and low activity was noted against *M. catarrhalis*, *E. faecalis*, *S. pneumoniae* and *M. smegmatis* (mean MIC values of 8.0 mg/ml). Some antimicrobial activity was seen against *C. neoformans* and *S. pyogenes* with mean MIC values of 3.0 and 4.0 mg/ml. Noteworthy activity was again noted against *S. agalactiae*, with a mean MIC value of 0.8 mg/ml.

∑FIC values for the combination of *A. afra* with *L. javanica* against *K. pneumoniae* and *M. catarrhalis* could not be calculated, as the end point MIC value of *L. javanica* was not determined. However, based on the MIC values a tentative interpretation is made. Thus against *K. pneumoniae* and *M. catarrhalis* the combination of *A. afra* with *L. javanica* aqueous extracts additive interactions are apparent. Additivity is also evident against *E. faecalis*, *C. neoformans*, *S. pyogenes* and *S. agalactiae* from the calculated ∑FIC values. Indifferent interactions were noted against *S. pneumoniae* and *M. smegmatis*.

The combination of *A. afra* with *L. javanica* aqueous extract has not been studied previously. In this study, low to poor activity was noted when testing this combination. ∑FIC values showed the combination was additive for most of the pathogens while some were indifferent. Interestingly *S. agalactiae* was the most sensitive pathogen to the combination. Thus, this

combination would be ideal in the treatment of *S. agalactiae* and *S. pyogenes* related respiratory infections.

3.2.2.2 Isobologram interpretation

A. afra, tested with *L. javanica* essential oils in combination against *M. catarrhalis* at different ratios were plotted on isobolograms. Results display additive interactions with all points on the line (Figure 3.4). The dichloromethane: methanol extracts of the same combination also yield additive interactions with two exceptions. The exceptions, are two points (ratios 2:8 (Figure 3.4, point a) and 1:9 (Figure 3.4, point b), below ∑FIC = 0.5, wherein *L. javanica* is in the majority. These two combinations display synergistic interactions. The 1:1 ratio of the combination is represented by a square (□) in the isobologram.

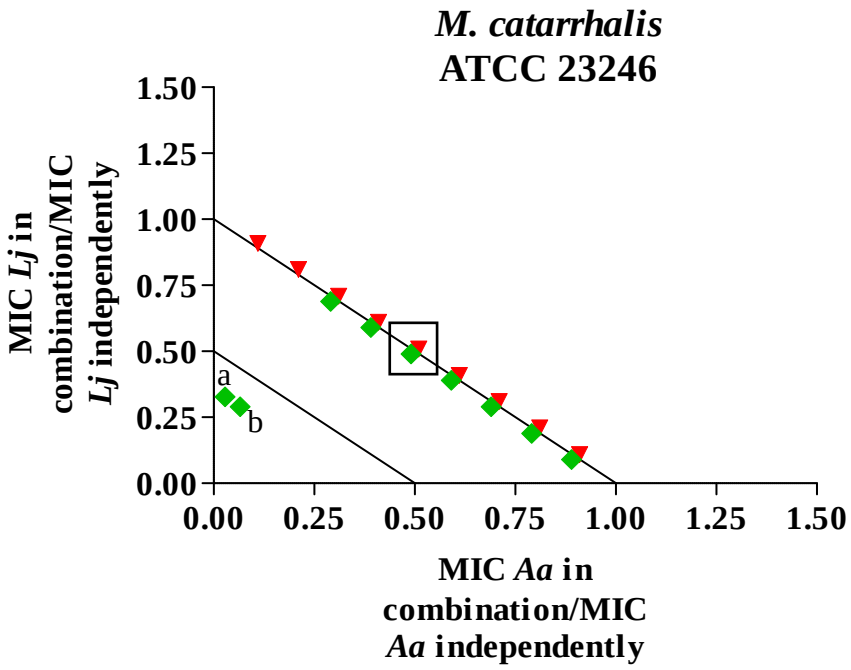


Figure 3.4 Isobologram of the combination of *A. afra* with *L. javanica* essential oils (▼) and the dichloromethane: methanol extracts (■) against *M. catarrhalis* (a – ratio 2:8; b – ratio 1:9); □→ 1:1 combination.

The aqueous extracts have not been included in the isobologram as the end point for *L. javanica* was not determined at the highest concentration tested, thus an isobologram could not be constructed. However, a bar graph as depicted in Figure 3.6 shows the MIC values of the different ratios compared to the MIC values of *A. afra* independently (8.0 mg/ml) and *L. javanica* (≥16.0 mg/ml) alone against *M. catarrhalis*. The MIC values of the combination of

A. afra with *L. javanica* at ratios 9:1 and 8:2 were consistent at 8.0 mg/ml. At the ratio of 7:3, the MIC value went up to 16.0 mg/ml and at 6:4, the MIC value noted was 12.0 mg/ml.

Against *K. pneumoniae* (Figure 3.5) the combination of *A. afra* with *L. javanica* essential oils yielded additive interactions for all the ratios tested. The dichloromethane: methanol extracts of the combination showed additive interactions for all ratios. The aqueous extracts could not be included in the isobologram as the end point of *L. javanica* could not be determined.

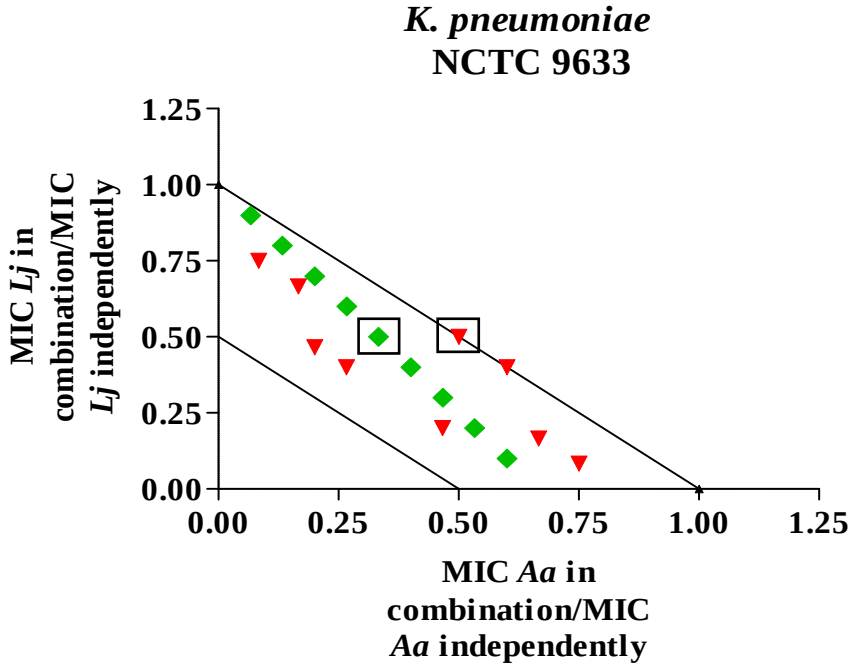


Figure 3.5 Isobologram of the combination of *A. afra* with *L. javanica* essential oils (▼) and the dichloromethane: methanol extracts (■) against *K. pneumoniae*; □ → 1:1 combination.

The bar graph of the MIC values obtained for the different ratios tested are shown in Figure 3.6. This shows that at higher concentrations of *A. afra*, between ratios 9:1, 8:2 and 6:4, the MIC values (6.0 mg/ml) of the combination is lower than that of *A. afra* alone or *L. javanica* alone. The MIC value of the ratio 7:3 was the same as *A. afra*, 8.0 mg/ml. When combined in equal ratios, the activity of the combination is the same as that of *A. afra* alone. However, when *L. javanica* is in the majority, the MIC of the combination was higher than the concentration of the combination that was tested i.e. ≥16.0 mg/ml. Where *A. afra* (aqueous extract) is present in higher concentrations, better antimicrobial activity is noted against *K. pneumoniae* (Figure 3.6).

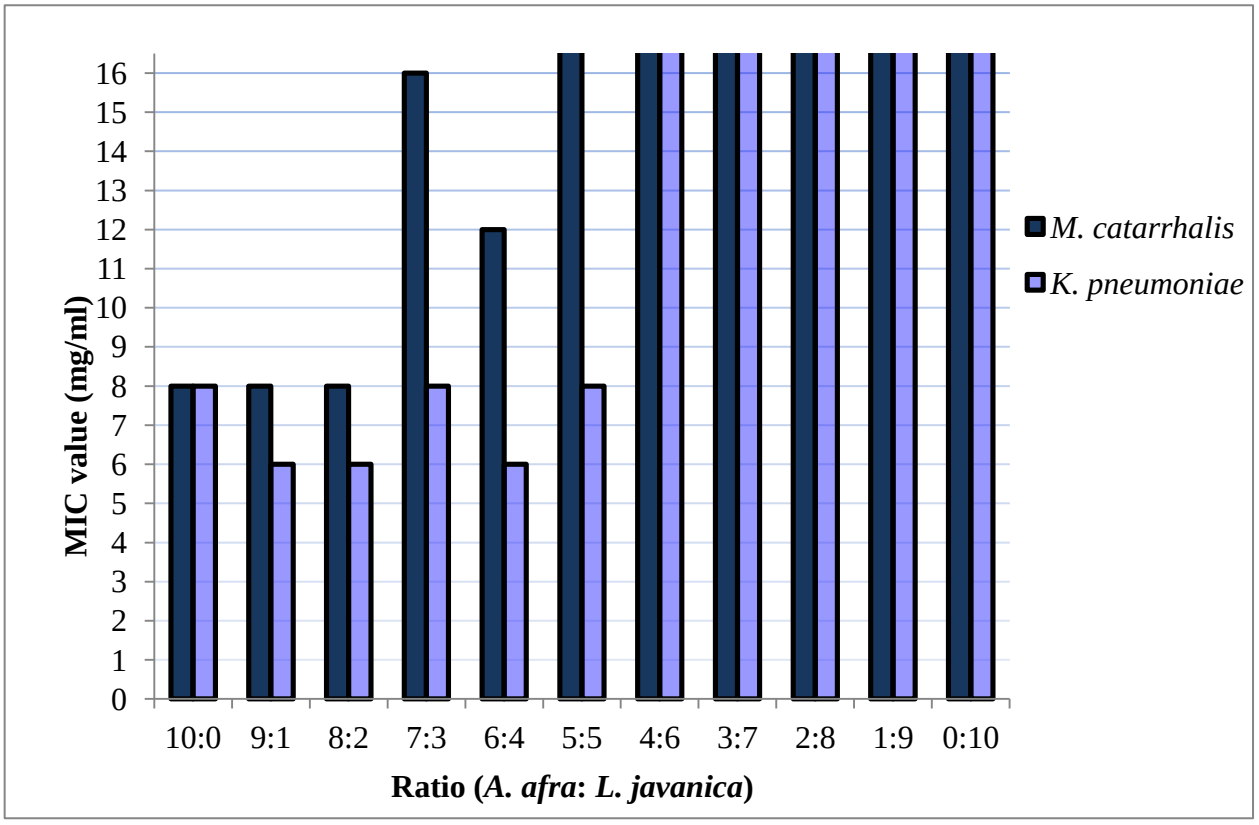


Figure 3.6 The MIC values of the different ratios of the combination of *A. afra* with *L. javanica* aqueous extracts against *M. catarrhalis* and *K. pneumoniae*.

In the combination of *A. afra* with *L. javanica* against *E. faecalis* (Figure 3.7), the essential oils showed additive pharmacological interaction for all the ratios tested. The dichloromethane: methanol extracts showed additive interactions as well for most of the ratios with the exception of two ratios. The ratio 2:8 (Figure 3.7, point c) showed additivity and the ratio 1:9 (Figure 3.7, point d) was synergistic. This suggests that an ideal combination of *A. afra* with *L. javanica* dichloromethane: methanol extracts would be with one part of *A. afra* and nine parts of *L. javanica*. The aqueous extracts showed additive interactions for all the ratios tested with one ratio i.e. 9:1, was found on the 1:0 and 0:1 line of the isobologram. *A. afra* was the majority plant in this combination.

Isobologram interactions of the combination of *A. afra* with *L. javanica* essential oils against *C. neoformans* (Figure 3.8) resulted in additive interactions. The dichloromethane: methanol extract also gave additivity at the ratios tested. Two ratios however, showed indifference when combined. These were the ratios 7:3 (Figure 3.8, point e), where *A. afra* was in the majority and 1:9 (Figure 3.8, point f), where *L. javanica* was dominant. When the aqueous

extracts of *A. afra* and *L. javanica* were combined in nine ratios, additive interactions were also noted

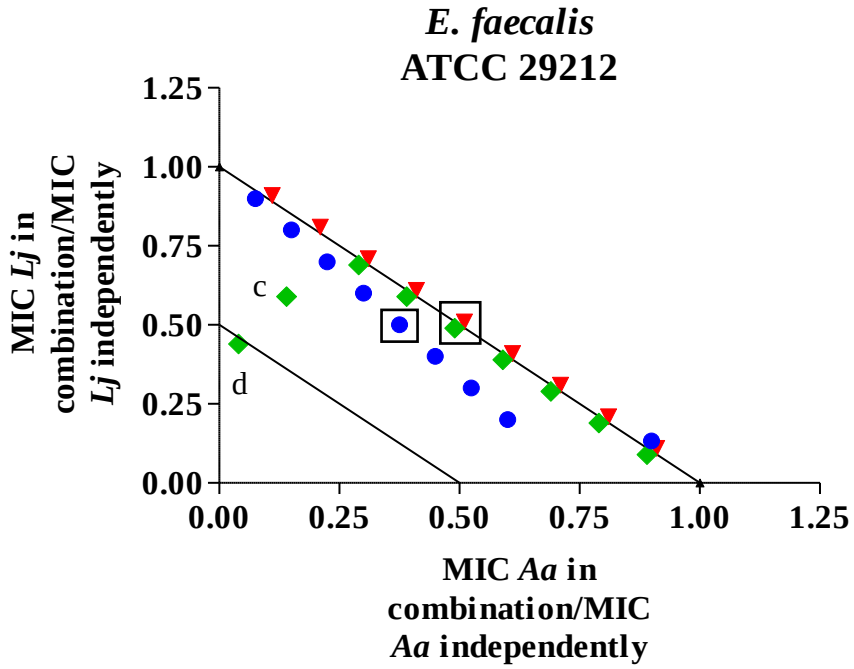


Figure 3.7 Isobologram of the combination of *A. afra* with *L. javanica* essential oils (▼), dichloromethane: methanol (■) and aqueous extracts (●) against *E. faecalis* (c – ratio 2:8; d – ratio 1:9); □ → 1:1 combination.

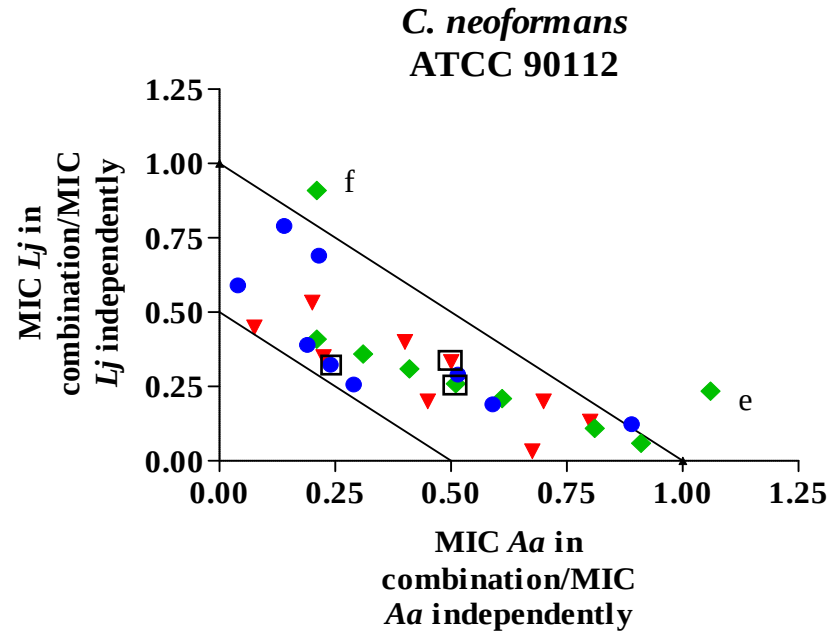


Figure 3.8 Isobologram of the combination of *A. afra* with *L. javanica* essential oils (▼) dichloromethane: methanol (■) and aqueous extracts (●) against *C. neoformans* (e – ratio 7:3; f – ratio 1:9); □ → 1:1 combination.

The 1:1 combination of *A. afra* with *L. javanica* essential oil and dichloromethane: methanol extracts of the isobolograms (Figure 3.4 and 3.5) and the ∑FIC values showed good

correlation and congruency against *M. catarrhalis* and *K. pneumoniae* (Table 3.4 and 3.5). Against *E. faecalis* and *C. neoformans*, similarities were apparent for the combination of the essential oils and the aqueous extracts. Partial congruency was noted with the differences in the dichloromethane: methanol extracts where additivity was seen in the isobolograms and indifference was seen in the ∑FIC values. One possible explanation for this variability is the possibility of disparate mechanisms of interaction due to the differing methods of testing (Beale and Sutherland, 1983; van Vuuren, 2007).

3.3 Conclusions

- The major constituents of the essential oil of *A. afra* were camphene (4.0%), 1,8-cineole (17.0%), artemisia ketone (6.4%), β-thujone (6.0%), camphor (41.0%) and borneol (8.6%), representing 83.0% accumulatively.
- The major compounds of the essential oil of *L. javanica* are 1,8-cineole (2.3%), *p*-cymene (3.4%), *trans*-linalool oxide (4.5%), *cis*-linalool oxide (4.0%), linalool (70.7%) and caryophyllene oxide (6.9%), representing accumulatively 91.8%.
- The TLC studies for *A. afra* and *L. javanica* essential oils, dichloromethane: methanol extracts and the aqueous extracts showed visible bands under different wavelengths and gave a unique fingerprint of the plants independently and in combination used in this assay.
- The dichloromethane: methanol extracts of *A. afra* indicated better antimicrobial activity (MIC values between 0.5-6.3 mg/ml) than that of *A. afra* essential oil (MIC values between 1.5-8.7 mg/ml) followed by the aqueous extracts with MIC values ranged from 1.7-12.0 mg/ml. The best activity for the dichloromethane: methanol extracts of *A. afra* was against *S. pneumoniae* with a MIC value of 0.5 mg/ml.
- The highest *efficac*y, using the ∑FIC determination method, in the combination study of *A. afra* with *L. javanica* was noted with the dichloromethane: methanol extracts against *S. agalactiae* (∑FIC value of 0.4) which was synergistic.
- Synergistic interactions in the isobologram study of the combination of *A. afra* with *L. javanica* were noted with the best activity noted in two ratios for the dichloromethane: methanol extracts against *M. catarrhalis*. One ratio against *E. faecalis* was also found to be synergistic
- No antagonism for the combination was noted against any of the pathogens tested.

- The efficacies shown against the pathogens tested, using the MIC micro-titre plate, ∑FIC determination and the isobologram methods give credence and provide evidence to support the traditional use of *A. afra* with *L. javanica* in combination for the treatment of *M. catarrhalis*, *E. faecalis*, and *S. agalactiae* related respiratory tract infections. It should, however, be noted that further toxicity and *in vivo* studies are needed to confirm safety and efficacy.

Chapter 4

The antimicrobial efficacy of *Artemisia afra* Jacq. Ex Willd and *Osmitopsis asteriscoides* (L.) Cass. In combination.

4.1 Introduction

The plants *A. afra* and *O. asteriscoides* have been mentioned in the ethnobotanical literature for their use in combination. Traditionally, a tincture of this combination has been used for the treatment of respiratory complaints (Watt and Breyer-Brandewijk, 1962). The aim of the study of this combination was to determine if any increase, decrease or non-inhibitory effect was noted when the two plants were combined. A monograph (medicinal uses, botanical description, geographical distribution and locality) for *O. asteriscoides* can be found in Appendix A7.

4.2 Results and discussion

4.2.1 Chromatographic techniques

4.2.1.1 Essential oil composition of *O. asteriscoides*

GC-MS results revealed a total of 32 compounds with the major compound as 1,8-cineole (59.0%). As shown in Table 4.1, camphor and α-terpineol were also present at 8.2 and 7.2% respectively. The major compounds accumulatively make up 74.4% of the total composition of the oil (91.6%).

In the study by Viljoen et al. (2003), where *O. asteriscoides* was collected from a population near Betty's Bay in the South Western Cape region of South Africa, the two major compounds found in the pooled sample of plants from the population were 1,8-cineole and camphor, representing 59.9% and 12.4% respectively of the total oil. Other minor compounds were also found. These included α-pinene, camphene, ρ-cymene, longipinene, and α-terpineol. In the current study the percentage of 1,8-cineole is congruent (59.0%) with that of Viljoen et al. (2003) and cited in van Vuuren and Viljoen (2006). The percentage of α-terpineol in this study (7.2%) is congruent with that of Viljoen et al, (2003) which contained 7.8% of the total essential oil. The percentage of camphor (8.2%) is slightly different to

Viljoen et al. (2003) which made up 12.4% of the total composition of oil. These differences in chemical composition are most likely due to the different collection sites.

Table 4.1 GC-MS profile of *O. asteriscoides*.

RRI*	Compounds	Area (%)
1016	α-Pinene	2.0
1019	α-Thujene	0.1
1057	Camphene	1.1
1104	β-Pinene	0.5
1117	Sabinene	1.2
1159	Myrcene	0.1
1193	α-Terpinene	0.3
1184	2,3-Dehydro-1,8-cineole	0.2
1193	Limonene	0.1
1202	**1,8-Cineole**	**59.0**
1242	γ-Terpinene	0.5
1270	*p*-Cymene	0.7
1281	Terpinolene	0.1
1382	(z)-Hex-3-en-1-ol	0.1
1441	*trans*-Linalool oxide	0.1
1456	*trans* Sabinene hydrate	0.2
1464	α-Longipinene	2.7
1479	α-Ylangene	0.1
1511	Chrysanthenone	0.4
1521	**Camphor**	**8.2**
1546	Linalool	0.4
1562	Terpineol-4-ol-acetate	0.2
1573	Pinocarvone	0.2
1586	β-Elemene	0.1
1602	Terpinen-4-ol	2.3
1675	d-Terpineol	0.4
1701	**α-Terpineol**	**7.2**
1709	Borneol	1.1
1745	Piperitol	0.1
1876	*cis* Carveol	0.1
2010	Caryophyllene oxide	0.4
2122	γ-Bicyclofarnesol	1.4
Total		**91.6**

*RRI: Relative retention indices calculated against *n*-alkanes.
% calculated from TIC data.

4.2.1.2 Thin layer chromatography

The thin layer chromatography of the essential oils (Figure 4.1) of *O. asteriscoides* individually (Figure 4.1, lane 2) and the combination with *A. afra* (Figure 4.1, Lane 3) at 254 nm showed some polar compounds. Compounds in *A. afra* alone (Figure 4.1, lane 1) are

visible in the combination as well. After derivatization, many more compounds were visible. Major bands at R_f = 0.2, 0.25, 0.4 and 0.45 were evident.

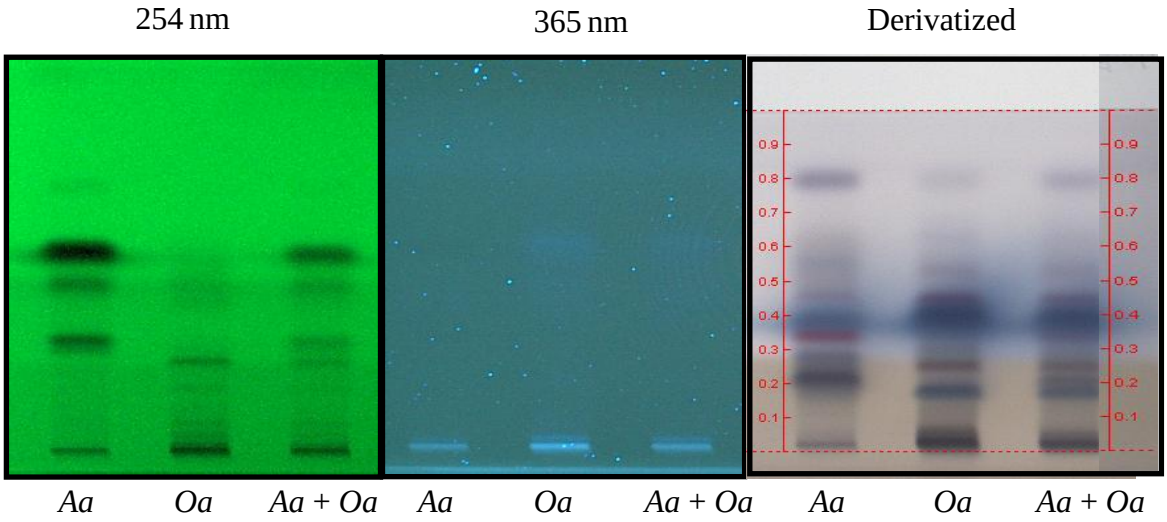


Figure 4.1 TLC of the essential oils of *A. afra*, *O. asteriscoides* individually and in combination at 254 nm, 365 nm and visualized with vanillin-sulphuric acid reagent. *Aa – A. afra*; *Oa – O. asteriscoides*; *Aa + Oa – A. afra* with *O. asteriscoides*.

The dichloromethane: methanol extracts showed that good separation was achieved as shown in Figure 4.2. Many compounds were visible under 254 nm. Under 365 nm, the highly fluorescent compounds at R_f = 0.3 and 0.35 (blue in colour) found in *A. afra* were also visible.

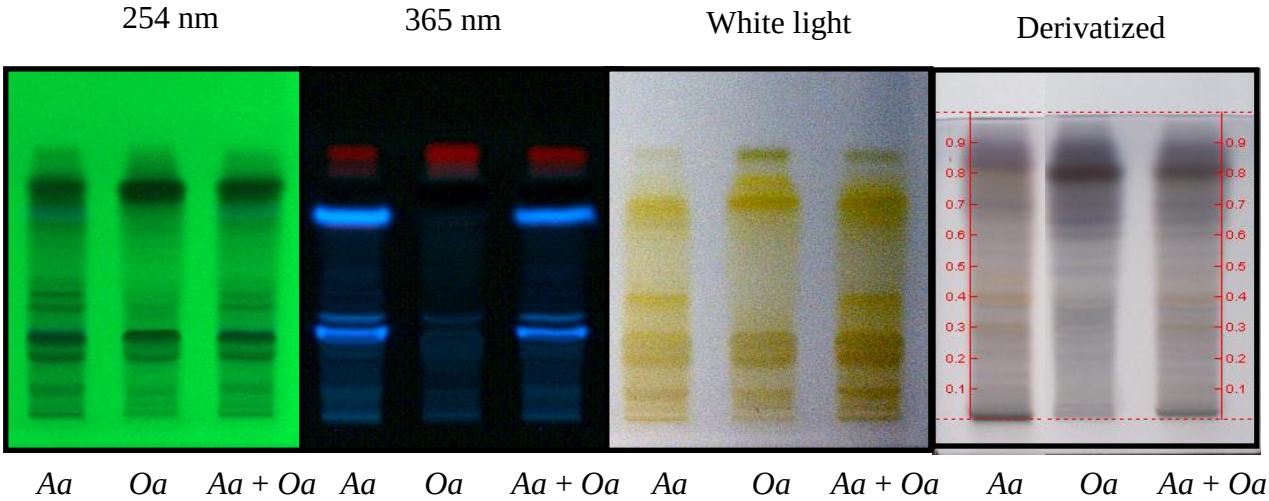


Figure 4.2 TLC of the dichloromethane: methanol extracts of *A. afra*, *O. asteriscoides* individually and in combination 254 nm, 365 nm, visualized under white light and visualized with vanillin-sulphuric acid reagent. *Aa – A. afra*; *Oa – O. asteriscoides*; *Aa + Oa – A. afra* with *O. asteriscoides*.

Scott et al. (2004), studied the dichloromethane extracts of *O. asteriscoides* and found four bands when derivatized at R_f = 0.25 (yellow), 0.39 (yellow), 0.58 (purple) and 0.73 (light purple. Similar bands were detected in this study with an extra major band at R_f = 0.8.

The aqueous extracts showed poor separation for *O. asteriscoides* and the combination with *A. afra*. A major band was visualized after derivatization at R_f = 0.9 for *O. asteriscoides* and the combination.

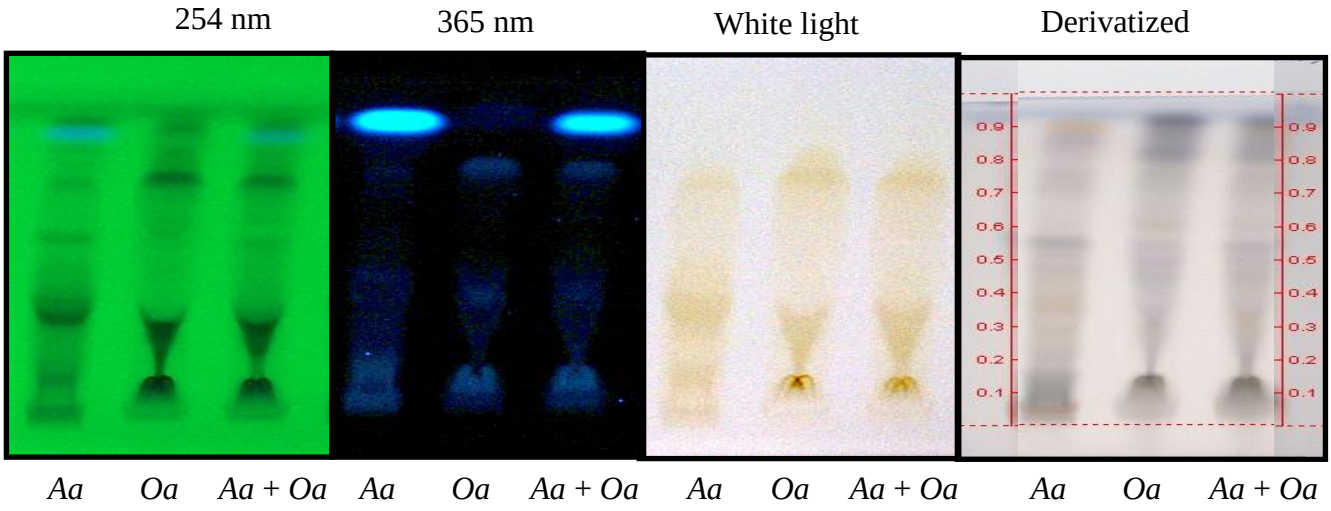


Figure 4.3 TLC of the aqueous extracts of *A. afra*, *O. asteriscoides* individually and in combination at 254 nm; 365 nm; visualized under white light; visualized with vanillin-sulphuric acid reagent. *Aa – A. afra*; *Oa – O. asteriscoides*; *Aa + Oa – A. afra* with *O. asteriscoides*.

4.2.2 Antimicrobial analysis

4.2.2.1 MIC assays and FIC determination

4.2.2.1.1 Essential oils

The MIC values of *O. asteriscoides* essential oils individually and in combination with *A. afra* are given in Table 4.2. Even though the MIC values of *A. afra* essential oil have been discussed and evaluated in Chapter 3, Section 3.2.2.1, they have been shown in Table 4.2 for the sake of completeness. MIC values for *O. asteriscoides* essential oil ranged from as low as 1.0 mg/ml to 8.0 mg/ml. Noteworthy activity was seen against *C. neoformans* (MIC value = 2.0 mg/ml), *S. pyogenes* (MIC value = 1.7 mg/ml) and against *M. smegmatis* (MIC value = 1.3 mg/ml). The best MIC value of 1.0 mg/ml was obtained for *O. asteriscoides* against *S. pneumoniae.*

Table 4.2 MIC (mg/ml) and ∑FIC values of *A. afra* and *O. asteriscoides* essential oils alone and in combination.

Micro-organism	MIC (mg/ml)			∑FIC	
	A. afra	***O. asteriscoides***	***A. afra* with *O. asteriscoides***	***A. afra* with *O. asteriscoides* (1:1)**	**Interpretation**
K. pneumoniae NCTC 9633	8.0	8.0	8.0	1.0	Additive
M. catarrhalis ATCC 23246	8.7	8.0	8.0	1.0	Additive
E. faecalis ATCC 29212	8.7	8.0	8.0	1.0	Additive
C. neoformans ATCC 90112	3.8	**2.0**	**2.0**	0.8	Additive
S. pneumoniae ATCC 49619	3.3	**1.0**	**1.0**	0.7	Additive
S. pyogenes ATCC 8668	**1.7**	**1.7**	**1.5**	0.9	Additive
S. agalactiae ATCC 55618	4.8	4.7	7.0	1.5	Indifferent
M. smegmatis (clinical)	**1.5**	**1.3**	**1.5**	1.1	Indifferent

*Values given in bold indicate noteworthy activity.
Shaded area: results previously discussed in Chapter 3 but included here for reference purposes.
Controls excluded for the sake of brevity. Please refer to Chapter 3 for further detail regarding control values.

Viljoen et al. (2003) investigated the antimicrobial activity of *O. asteriscoides* essential oil using the disk diffusion, MIC and time-kill studies. MIC results for *E. faecalis* showed no activity at the highest concentration tested and an MIC value of 8.0 mg/ml against *C. neoformans* was obtained. The antimicrobial activity of *O. asteriscoides* is said to be mostly due to the high percentage of 1,8-cineole, which has been shown to have a broad spectrum of antimicrobial activity (Pattnaik et al., 1997). Historically cineole rich essential oils have been used in the treatment of respiratory conditions (Silvestre et al., 1999; Viljoen et al., 2003). Camphor, which is also a major compound found in *O. asteriscoides*, is known to be an antiseptic and a decongestant (Bruneton, 1995). Viljoen et al. (2003) tested the antimicrobial activity of 1,8-cineole and camphor independently and in combination and compared it to *O.*

asteriscoides essential oil and found that the two compounds act synergistically to enhance the overall activity of the oil.

O. asteriscoides essential oil was tested by van Vuuren and Viljoen (2006) using the MIC micro-titre plate method and time-kill studies. MIC values were determined against *S. aureus*, *S. epidermidis*, *B. cereus*, *B. subtilis*, *E. coli*, *P. aeruginosa*, *E. faecalis*, *K. pneumoniae*, *C. albicans* and *C. neoformans*. Comparable pathogens, *E. faecalis*, *K. pneumoniae* and *C. neoformans* gave MIC values of 7.0, 11.9 and 1.0 mg/ml respectively. These results are in alignment with those obtained in the present study. Time-kill studies revealed bactericidal activity against *S. aureus* after 24 hrs. Against *K. pneumoniae* cidal activity was noted within one hour of exposure; however, regrowth occurred after four hours. Cidal effects were noted against *C. albicans* with four hours of exposure to the essential oil.

In the study by van Vuuren (2007), MIC assays were performed on *O. asteriscoides* essential oil against ten pathogens. Two of these i.e. *E. faecalis* and *C. neoformans*, can be compared to the results obtained in this study. MIC values obtained by van Vuuren (2007) were 8.0 mg/ml for both pathogens. The results of this study are comparable with that of van Vuuren (2007) for the pathogen *E. faecalis*. However, for *C. neoformans*, the MIC determined in this study was much lower (2.0 mg/ml) . A possible explanation could be the difference in the chemical composition of the samples an due to the fact that the localities for sample collection were different, thus producing a different MIC.

In the combination of *O. asteriscoides* with *A. afra*, noteworthy antimicrobial activity was noted against the same pathogens as was for *O. asteriscoides* i.e. *C. neoformans* (2.0 mg/ml), *S. pneumoniae* (1.0 mg/ml), *S. pyogenes* (1.5 mg/ml) and *M. smegmatis* (1.5 mg/ml). The MIC values of the combination against *K. pneumoniae*, *M. catarrhalis* and *E. faecalis* remained constant for the essential oils independently and in combination. Thus, the ∑FIC values showed additive interactions when *A. afra* was combined with *O. asteriscoides* against these pathogens (∑FIC = 1). Other ∑FIC values of note were against *C. neoformans*, *S. pneumoniae* and *S. pyogenes* with values of 0.8, 0.7 and 0.9 respectively. Indifferent interactions were noted against *S. agalactiae* and *M. smegmatis*. No antagonism was noted for the combination against any of the pathogens tested.

4.2.2.1.2 Dichloromethane: methanol extracts

The *O. asteriscoides* dichloromethane: methanol extract was active against all the pathogens tested (Table 4.3) with at least some antimicrobial activity noted (MIC values between 0.3 - 4.0 mg/ml). Activities for *S. pneumoniae* and *S. pyogenes* showed noteworthy sensitivity both with MIC values of 0.4 mg/ml, indicating *O. asteriscoides* is a strong inhibitor against these pathogens. The best activity was noted against *C. neoformans* where an MIC value of 0.3 mg/ml was obtained.

Table 4.3 MIC and ∑FIC values of *A. afra* and *O. asteriscoides* dichloromethane: methanol extracts alone and in combination.

Micro-organism	MIC (mg/ml)			∑FIC	
	A. afra	***O. asteriscoides***	***A. afra* with *O. asteriscoides***	***A. afra* with *O. asteriscoides* (1:1)**	**Interpretation**
K. pneumoniae NCTC 9633	6.3	4.0	6.0	1.2	Indifferent
M. catarrhalis ATCC 23246	4.7	4.0	4.0	0.9	Additive
E. faecalis ATCC 29212	6.3	2.0	4.0	1.3	Indifferent
C. neoformans ATCC 90112	1.2	**0.3**	**0.3**	0.7	Additive
S. pneumoniae ATCC 49619	**0.5**	**0.4**	**0.1**	**0.3**	**Synergy**
S. pyogenes ATCC 8668	1.1	**0.4**	**0.1**	**0.2**	**Synergy**
S. agalactiae ATCC 55618	3.0	1.3	1.0	0.6	Additive
M. smegmatis (clinical)	1.7	1.4	**0.5**	**0.3**	**Synergy**

*Values given in bold indicate noteworthy activity.
Shaded area: results previously discussed in Chapter 3 but included here for reference purposes.
Controls excluded for the sake of brevity. Please refer to Chapter 3 for further detail regarding control values.

For the combination with *A. afra*, some antimicrobial activity was noted against *K. pneumoniae*, *M. catarrhalis*, *E. faecalis* and *S. agalactiae* (MIC values between 1.0 - 6.0 mg/ml). Noteworthy activity was seen against *C. neoformans*, *S. pneumoniae*, *S. pyogenes* and *M. smegmatis*. *A. afra* with *O. asteriscoides* in combination were strong inhibitors against these four pathogens with MIC values ranging at 0.1 - 0.5 mg/ml.

∑FIC values then calculated showed synergistic interactions with the combination against *S. pneumoniae*, *S. pyogenes* and *M. smegmatis* with values of 0.3, 0.2 and 0.3 respectively. Additive interactions were observed against *M. catarrhalis*, *C. neoformans* and *S. agalactiae*. Thus against all the pathogens tested for this combination, either synergy or additivity was noted with the exception of *K. pneumoniae* and *E. faecalis* which were indifferent.

The dichloromethane: methanol extracts of *O. asteriscoides* have not to this date been tested for their antimicrobial activity. This study demonstrates for the first time the antimicrobial activity, using MIC methodology, of the dichloromethane: methanol extracts of *O. asteriscoides* independently and in combination. It is surprising that no studies have been done on the extracts as the traditional method of administration of *O. asteriscoides* is as a tincture. It is thus important that the alcohol extracts be tested for their activity in treating respiratory tract diseases.

4.2.2.1.3 Aqueous extracts

The antimicrobial analysis of the aqueous extracts (Table 4.4) of *O. asteriscoides* yielded MIC values between 0.5 - 9.0 mg/ml against the pathogens tested. The lowest MIC value was against *C. neoformans*, with a value of 0.5 mg/ml. Moderate inhibition was also noted for *O. asteriscoides* against *S. pneumoniae* and *S. agalactiae* with values of 1.5 mg/ml. Against *S. pyogenes* the MIC value obtained was 1.8 mg/ml.

For the combination of *A. afra* with *O. asteriscoides* some antimicrobial activity was obtained against *S. pneumoniae* and *S. agalactiae* with MIC values of 2.0 mg/ml, and 4.0 mg/ml against *M. smegmatis*. Moderate antimicrobial activity was recorded for *S. pyogenes* with an MIC value of 1.5 mg/ml. Strong noteworthy inhibitory activity and the lowest MIC value (0.5 mg/ml) was obtained against *C. neoformans*.

∑FIC values obtained for the combination of *A. afra* with *O. asteriscoides* aqueous extracts showed additive interactions against *K. pneumoniae*, *M. catarrhalis*, *E. faecalis*, *C. neoformans*, *S. pneumoniae*, *S. pyogenes* and *M. smegmatis*. It is interesting to note none of the interactions of *A. afra* with *O. asteriscoides* yielded antagonistic effects.

Table 4.4 MIC and ∑FIC values of *A. afra* and *O. asteriscoides* aqueous extracts alone and in combination.

Micro-organism	MIC (mg/ml)			∑FIC	
	A. afra	***O. asteriscoides***	***A. afra* with *O. asteriscoides***	***A. afra* with *O. asteriscoides* (1:1)**	**Interpretation**
K. pneumoniae NCTC 9633	12.0	8.0	8.0	0.8	Additive
M. catarrhalis ATCC 23246	8.0	8.0	8.0	1.0	Additive
E. faecalis ATCC 29212	7.0	9.0	8.0	1.0	Additive
C. neoformans ATCC 90112	4.0	**0.5**	**0.5**	0.6	Additive
S. pneumoniae ATCC 49619	8.0	1.5	2.0	0.8	Additive
S. pyogenes ATCC 8668	5.3	1.8	1.5	0.6	Additive
S. agalactiae ATCC 55618	1.7	1.5	2.0	1.3	Indifferent
M. smegmatis (clinical)	8.0	4.0	4.0	0.8	Additive

*Values given in bold indicate noteworthy activity.
Shaded area: results previously discussed in Chapter 3 but included here for reference purposes.
Controls excluded for the sake of brevity. Please refer to Chapter 3 for further detail regarding control values.

Scott et al. (2004) performed disc diffusion assays on the aqueous extracts of *O. asteriscoides*. Pathogens tested included *S. aureus*, *P. aeruginosa*, *C. albicans* and *M. smegmatis*. In their study, no activity of the aqueous infusion was noted against any of the pathogens tested.

4.2.2.2 Isobologram interpretation

The isobologram of the combination of *A. afra* with *O. asteriscoides* essential oils, dichloromethane: methanol extracts and aqueous extracts against *M. catarrhalis* is shown in Figure 4.4. The essential oils show additive interactions for all the ratios tested. The dichloromethane: methanol extracts showed additive interactions for most of the ratios tested with two points as exceptions. These two points are synergistic and they are the ratios 1:9 (Figure 4.4, a) and 2:8 (Figure 4.4, b).

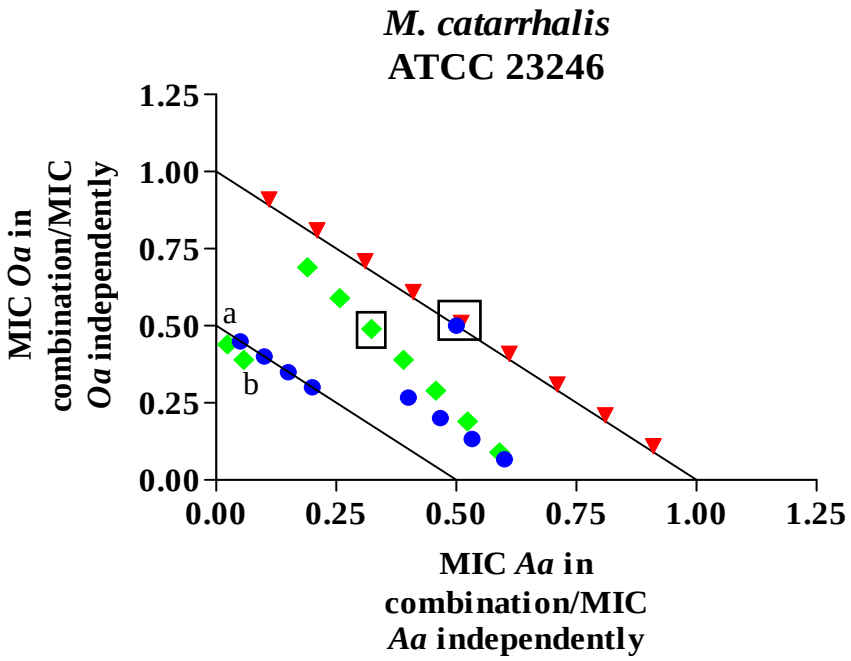


Figure 4.4 Isobologram of the combination of *A. afra* and *O. asteriscoides* essential oils (▼), dichloromethane: methanol extracts (■) and aqueous extracts (●) against *M. catarrhalis* (a – ratio 1:9; b – ratio 2:8); □→ 1:1combination.

This synergy displayed between *A. afra* with *O. asteriscoides* takes place when the ratio of *O. asteriscoides* is in the majority. The aqueous extracts showed additive pharmacological interactions for five ratios tested. Four ratios (4:6, 3:7, 2:8 and 1:9) wherein *O. asteriscoides* dominates in the combination were synergistic. The 1:1 combination of the essential oils, dichloromethane: methanol extracts and the aqueous extracts are shown and depicted in Figure 4.4.

The ∑FIC values obtained for the 1:1 combination of the essential oils (Table 4.2), the dichloromethane: methanol extracts (Table 4.3) and the aqueous extracts (Table 4.4) against *M. catarrhalis* correlates with those results obtained in the isobolograms.

The interactions of the combination of *A. afra* with *O. asteriscoides* against *K. pneumoniae* are graphically represented in Figure 4.5. The essential oils demonstrate additive interactions for all the ratios tested. The dichloromethane: methanol extracts show mainly indifferent interactions for six of the nine ratios tested. Three of the ratios i.e. 4:6, 3:7 and 1:9 were additive. Thus, when *O. asteriscoides* is in the majority in the combination, additive interactions are found. The aqueous extracts show additive interactions with all the ratios

tested except one, which was synergistic. The ratio showing synergy is depicted in Figure 4.5, (c) is when *A. afra* is in the majority at a ratio of 8:2.

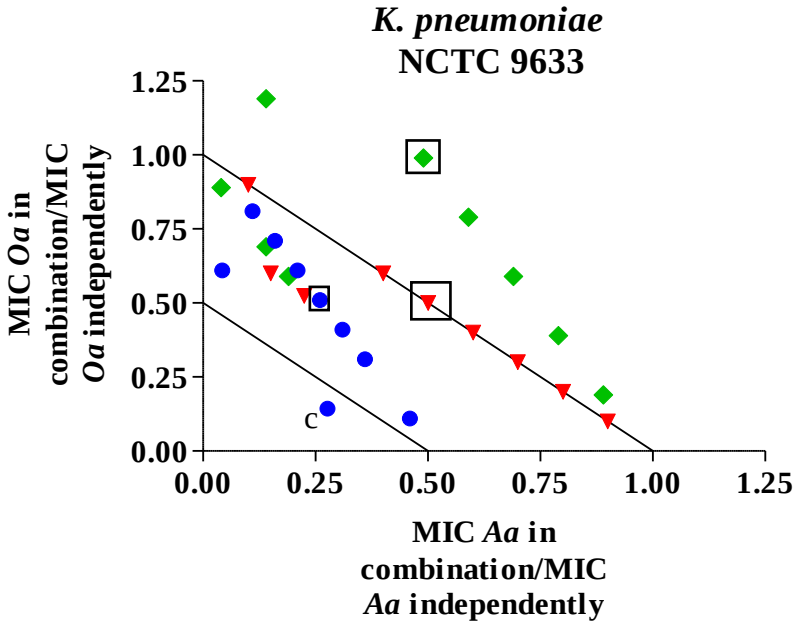


Figure 4.5 Isobologram of the combination of *A. afra* and *O. asteriscoides* essential oils (▼), dichloromethane: methanol extracts (■) and aqueous extracts (●) against *K. pneumoniae* (c – ratio 8:2); □ →1:1 combination.

The ∑FIC values of the essential oils (Table 4.2), the dichloromethane: methanol extracts (Table 4.3) as well as the aqueous extract (Table 4.4) of the 1:1 combination against *K. pneumoniae* correlates well with the isobologram (Figure 4.5).

The essential oils of the combination of *A. afra* with *O. asteriscoides* against *E. faecalis* are shown in Figure 4.6. All ratios for the combination showed additive interactions. The dichloromethane: methanol extracts showed additive interactions for most ratios tested. Two ratios tested were indifferent. These were at ratios 6:4 (where *A. afra* is dominant) and the 1:1 combination. The aqueous extracts showed additive interactions for all ratios except one (ratio 9:1) where *A. afra* was the dominant plant (Figure 4.6, d). This is interesting to note, as this information is not detected in the ∑FIC determination method wherein only 1:1 combinations are interpreted. ∑FIC data (Table 4.2, 4.3 and 4.4) and isobologram (Figure 4.6, 4.7 and 4.8) correlation was noted for the essential oils, dichloromethane: methanol and the aqueous extracts of the combination against *E. faecalis*.

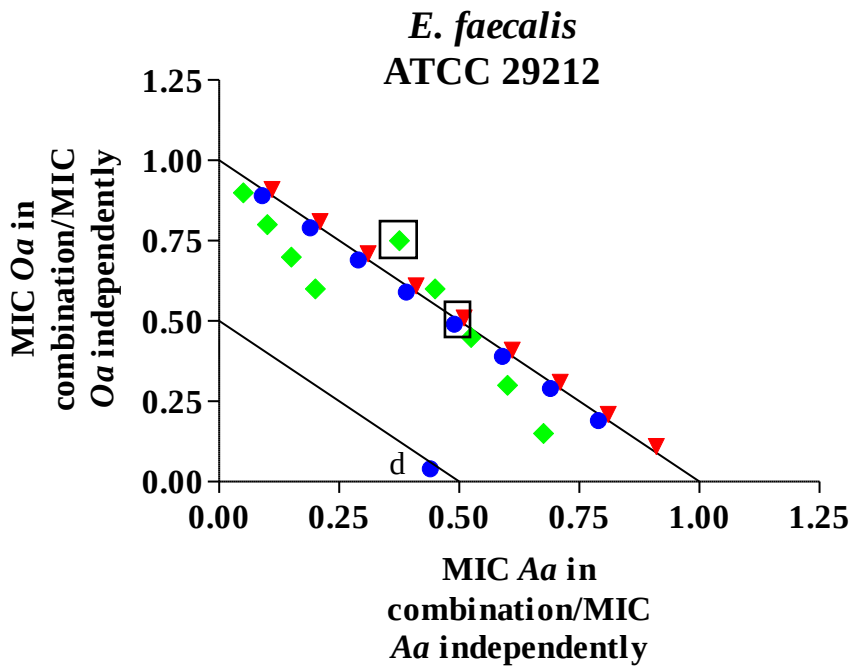


Figure 4.6 Isobologram of the combination of *A. afra* and *O. asteriscoides* essential oils (▼), dichloromethane: methanol extracts (■) and aqueous extracts (●) against *E. faecalis* (d – ratio 9:1); □ → 1:1 combination.

The interaction of *A. afra* and *O. asteriscoides* against *C. neoformans* is depicted in Figure 4.7. Results show the essential oil of the combination are additive for all the ratios tested except one which was synergistic (∑FIC = 0.5). This was the ratio 8:2 (Figure 4.7, e), where *A. afra* essential oil is the dominant oil in the combination. The dichloromethane: methanol extracts displayed a mix of additive and indifferent interactions. The ratios in which indifference was noted were at 4:6, 3:7, 2:8 and 1:9. These are all ratios in which *O. asteriscoides* is in the majority.

It is interesting to note that when *A. afra* and *O. asteriscoides* dichloromethane: methanol extracts are combined in equal parts, the interaction is additive. However, when the ratio of *O. asteriscoides* increases, the combination becomes indifferent. This is important to note as the traditional method of administration is a tincture. When traditional healers are mixing the combination together and if *O. asteriscoides* becomes more than *A. afra*, the administration of this combination could become less effective when treating *C. neoformans* related infections. It is reported that *O. asteriscoides* contains sesquiterpene lactones (Scott and Springfield, 2004), which may contribute to the possible toxicity. Thus, the possible harm is not due to any antagonism of the combination, but is from the possible toxicity and allergic

reactions of the sesquiterpene lactones present in *O. asteriscoides* (Scott and Springfield, 2004).

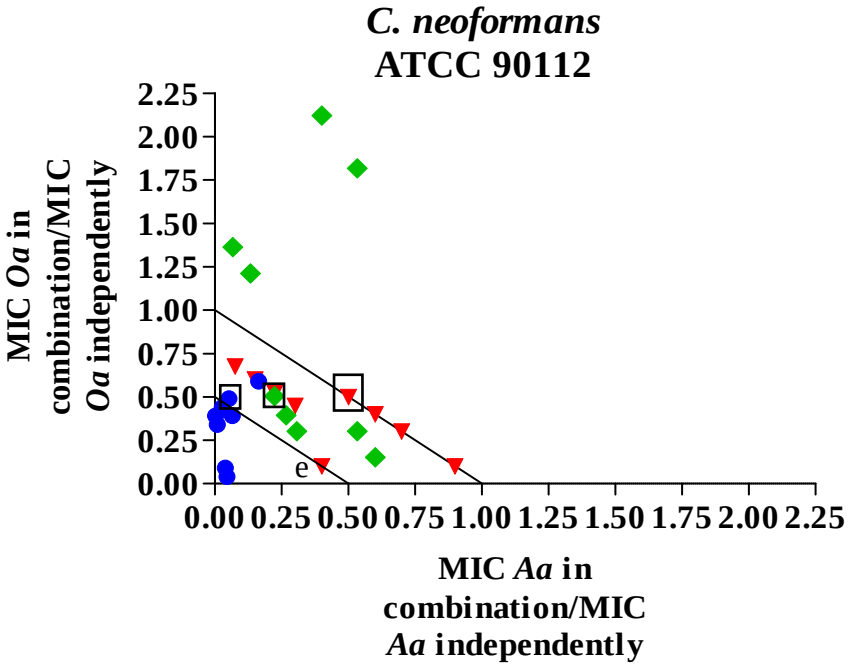


Figure 4.7 Isobologram of the combination of *A. afra* and *O. asteriscoides* essential oils (▼), dichloromethane: methanol extracts (■) and aqueous extracts (●) against *C. neoformans* (e – ratio 8:2); □ → 1:1 combination.

When the aqueous extracts of *A. afra* and *O. asteriscoides* were combined against *C. neoformans*, additive and synergistic interactions were noted. Ratios 9:1, 8:2, 6:4, 4:6, 3:7, 2:8 and 1:9 were the synergistic combinations. It is surprising that the aqueous extracts have produced such good synergy and the dichloromethane: methanol extracts have resulted in indifferent activity.

The ∑FIC values obtained and the isobologram were congruent for the combination of the essential oils, dichloromethane: methanol extracts and the aqueous extracts against *C. neoformans*.

Typically, the alcohol extracts produce better antimicrobial activity than the aqueous extracts. However, the combination is also administered as an infusion (Scott and Springfield, 2004) and although previous studies (Scott et al., 2004) have found no activity for the aqueous extracts, the results obtained in this study serves to validate the use of the combination of *A. afra* with *O. asteriscoides* in the form if an infusion.

4.3 Conclusions

- The major compounds present in the essential oil of *O. asteriscoides* were 1,8-cineole (59.0%), camphor (8.2%) and α-terpineol (7.2%) making up 74.4% of the total composition of the oil (91.6%).
- The TLC studies of *O. asteriscoides* (essential oils, dichloromethane: methanol and the aqueous extracts) showed visible bands under different wavelengths of light and gave a rather characteristic fingerprint of the plants used in this assay.
- The best antimicrobial activity for the essential oil of *O. asteriscoides* was obtained against *S. pneumoniae* with a mean MIC value of 1.0 mg/ml. For the dichloromethane: methanol extracts, noteworthy inhibitory activity with MIC values of 0.33 mg/ml was obtained against *C. neoformans*, followed by *S. pneumoniae* and *S. pyogenes* (MIC values = 0.4 mg/ml. The best activity for the aqueous extract of *O. asteriscoides* was obtained against *C. neoformans* (MIC value of 0.5 mg/ml).
- The most prominent antimicrobial activity of the combination of *A. afra* with *O. asteriscoides* essential oils was obtained against *S. pneumoniae* (MIC value of 1.0 mg/ml) For the dichloromethane: methanol extracts, the most interesting and noteworthy activity was obtained against *S. pneumoniae* and *S. agalactiae* with MIC values of 0.1 mg/ml. The combination of the aqueous extracts gave the best noteworthy activity against *C. neoformans* (MIC value of 0.5 mg/ml).
- In the combination of the essential oils of *A. afra* and *O. asteriscoides*, ∑FIC results revealed indifferent and additive pharmacological interaction. The dichloromethane: methanol extracts ∑FIC results showed synergistic interactions against *S. pneumoniae, S. pyogenes* and *M. smegmatis*. The aqueous extracts gave various ∑FIC results with additivity and indifference.
- Isobologram results of the combination show mainly additive interactions with the essential oils, with one point wherein eight parts of *A. afra* and two parts *O. asteriscoides* were combined (Figure 4.7, e) was synergistic.
- Synergistic interactions were found when tested against *M. catarrhalis* with the dichloromethane: methanol extracts at ratios 1:9 and 2:8 (Figure 4.4, a and b). The aqueous extracts showed synergy at ratios 4:6, 3:7, 2:8 and 1:9 against *M. catarrhalis*.
- Against *K. pneumoniae*, synergy was noted for one ratio i.e. at eight parts *A. afra* and two parts *O. asteriscoides* (Figure 4.5, c) of the combination of the aqueous extracts.

- The aqueous extracts showed synergy against *E. faecalis* at nine parts *A. afra* and one part *O. asteriscoides* (Figure 4.6, d).
- The best activity was obtained for the combination of *A. afra* with *O. asteriscoides* aqueous extracts against *C. neoformans*, where seven of the nine ratios tested were synergistic (Figure 4.7).
- No antagonism was noted for the combination against any of the pathogens tested.
- The outcomes of this chapter and the testing of the combination of *A. afra* with *O. asteriscoides* dichloromethane: methanol extracts provides *in vitro* evidence to support the use of a tincture in traditional African healing for the treatment of respiratory tract infections. Moreover, the aqueous extracts have also provided *in vitro* evidence of efficaciousness in the traditional use of the combination of *A. afra* with *O. asteriscoides*.

Chapter 5

The antimicrobial efficacy of *Artemisia afra* Jacq. Ex Willd and *Agathosma betulina* (P.J. Berguis) Pillans in combination.

5.1 Introduction

A. betulina is said to be one of the best-known medicinal plants of Southern Africa, which is used both locally and internationally for the treatment of various diseases (Moolla et al., 2007; Moolla and Viljoen, 2008). Traditionally, *A. betulina* is used in combination with *A. afra* and taken for colds and influenza or as a general tonic (Scott and Springfield, 2004). Traditionally 1% (15 mg/ml) of buchu extract is added in combination with *A. afra* (1.5% (10 mg/ml) of an extract) to a white sweet wine to make "The South African herbal wine" (Watt and Breyer-Brandwijk, 1962). Buchu and African wormwood have been studies extensively independently; however, the combination of the plants has never been evaluated scientifically. These two plants are widely used individually, generally well-accepted and respected plant medicines. Thus the reason of adding this combination to this study.

5.2 Results and discussion

5.2.1 Chromatographic techniques

5.2.1.1Essential oil composition of *A. betulina*

The essential oil composition of buchu revealed a total number of 30 compounds making up 97.5% of the total composition (Table 5.1). The major compounds were identified as limonene (18.9%), menthone (10.0%), isomenthone (31.4%), pseudodiosphenol (7.5%), and diosphenol (8.1%).

Previous studies on the chemical composition of buchu oil are discussed in Appendix A1. In brief, Kaiser et al. (1975) identified the main constituent as isomethone (50-60%) of the essential oil of buchu. Viljoen et al. (2006a) identified the major compound in commercial buchu oil purchased from Afriplex®, South Africa as menthone (29.2%). Limonene was also present (23.7%). The main constituent found in *A. betulina* essential oil in this study, was isomethone at 31.4%. Smaller quantities of menthone (10.0%) were found. Pulegone, a highly toxic compound noted by Viljoen et al. (2006a) at 8.4%, was also found in this study but at lower concentrations (4.1%).

Table 5.1 Essential oil composition of *A. betulina.*

RRI*	Compounds	Area (%)
1016	α-Pinene	0.8
1104	β-Pinene.	0.3
1117	Sabinene	0.8
1159	Myrcene	1.5
1192	α-Terpinene	0.1
1193	**Limonene**	**18.9**
1202	1,8-Cineole	1.7
1242	γ-Terpinene	tr
1250	(E)-β-Ocimene	0.5
1270	*p*-Cymene	0.5
1475	**Menthone**	**10.0**
1468	Fenchyl acetate	0.1
1496	**Isomenthone**	**31.4**
1546	Linalool	0.5
1572	Neoisopulegol	0.1
1577	*cis* Isopulegone	3.3
1587	*trans* Isopulegone	2.8
1602	Terpinen-4-ol	0.4
1610	Dihydrocarvone	0.2
1653	Pulegone	4.1
1692	Myrtenyl acetate	0.1
1701	α-Terpineol	0.1
1715	Germacrene D	0.1
1739	Piperitone	0.2
1752	**Pseudodiosphenol**	**7.5**
1825	**Diosphenol**	**8.1**
1873	*trans*-8-Mercapto-p-mentha-3-one	0.4
1905	*cis*-8-Mercapto-p-mentha-3-one	2.3
2082	Methyl eugenol	0.5
2188	Eugenol	0.2
Total		**97.5**

*RRI: Relative retention indices calculated against *n*-alkanes.
% calculated from TIC data.

From the literature, there are many other reports on the chemical composition of the essential oil of *A. betulina* (Gentry, 1961; Watt and Breyer-Brandwijk, 1962; Kaiser et al., 1975; Simpson, 1998; Lis-Balchin et al., 2001; van Wyk and Wink, 2004; Moolla, 2006; Viljoen et al., 2006a; Moolla et al., 2007; Moolla and Viljoen, 2008; van Wyk et al., 2009;). However, interest is only recently being directed towards the study of the non-volatile fraction of this

plant (Moolla et al., 2007; Vermaak et al., 2009). The importance of studying a plant in its entirety cannot be emphasized enough as the possibility of interactions of volatile and non-volatile compounds within a plant need to be considered as well as the individual contributions of each component (van Vuuren and Viljoen, 2009).

5.2.1.2 Thin layer chromatography

Thin layer chromatography analysis of the essential oils and the dichloromethane: methanol extracts of *A. afra* (discussed in Chapter 3), *A. betulina* and the combination of the two, revealed the presence of fluorescent compounds, which were visible under UV light of 254 nm as well as 365 nm. The aqueous extracts did not show good separation of the compounds and thus were not included. Figure 5.1 shows the chromatograms derived for the essential oils. A black fluorescent compound in *A. betulina*, at $R_f = 0.5$ was seen at 254 nm as well as at 365 nm (lighter in colour). Another major compound was detected in *A. betulina* at 365 nm at $R_f = 0.6$. When derivatized these compounds and various others were noted.

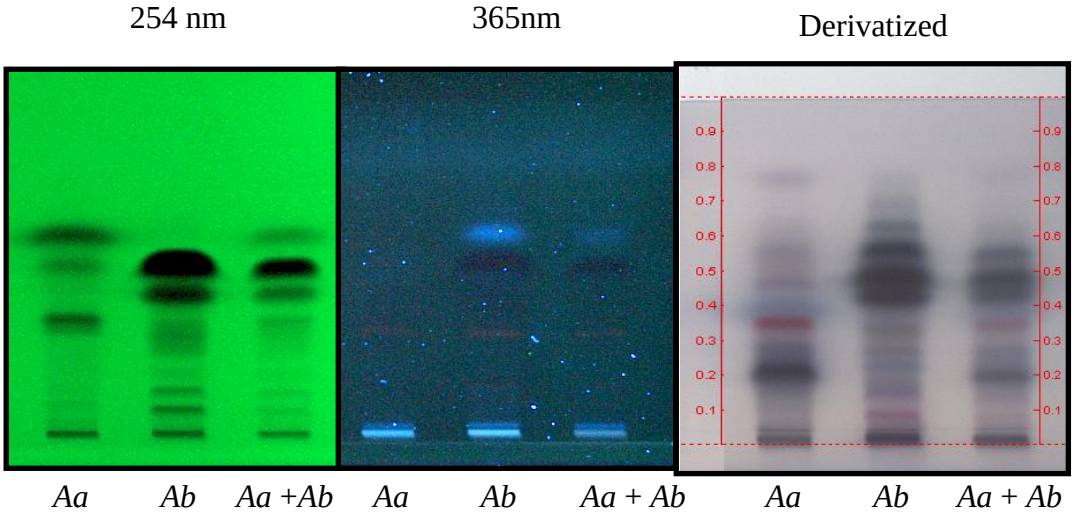


Figure 5.1 TLC fingerprints of the essential oils of *A. afra* and *A. betulina* alone and in combination at 254 nm, 365 nm and visualized with vanillin-sulphuric acid reagent. *Aa – A. afra*; *Ab – A. betulina*; *Aa + Ab – A. afra* with *A. betulina*.

The dichloromethane: methanol extracts (Figure 5.2) showed the presence of many compounds in *A. betulina* and in the combination with *A. afra*. At 254 nm major compounds were seen at $R_f = 0.65$ and $R_f = 0.8$. At 365 nm a compound showing up red was detected with an $R_f = 0.65$ as well. Under white light, the same compound is detected with the same R_f value. When derivatized the compound is not as visible but is mixed in with other visualized compounds.

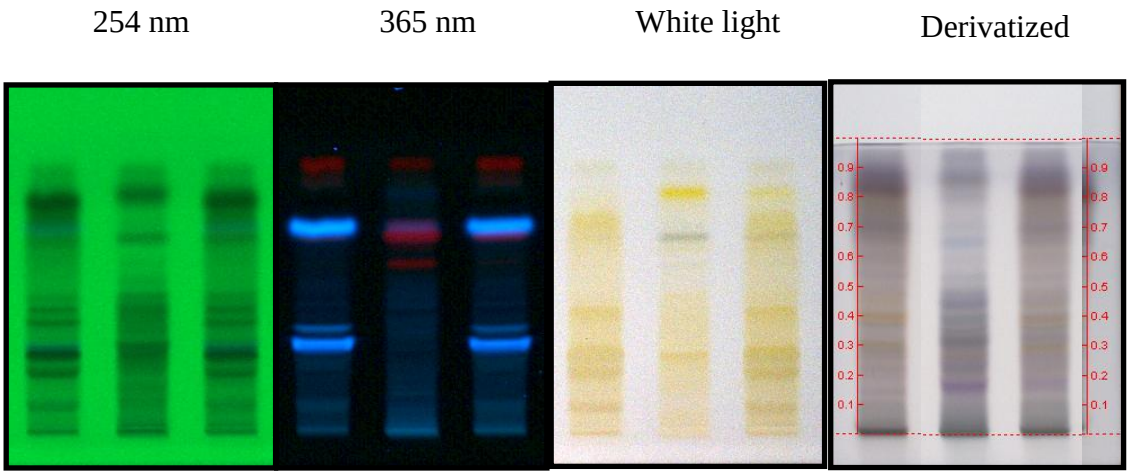


Figure 5.2 TLC fingerprints of the dichloromethane: methanol extracts of *A. afra* and *A. betulina* alone and in combination at 254 nm, 365 nm, visualized under white light and visualized with vanillin-sulphuric acid reagent. *Aa – A. afra*; *Ab – A. betulina*; *Aa + Ab – A. afra* with *A. betulina*.

5.2.2 Antimicrobial analysis

5.2.2.1 MIC assays and FIC determination

5.2.2.1.1 Essential oils

The MIC values and of the essential oil of *A. betulina* individually and in combination with *A. afra* are given in Table 5.2. The MIC values of *A. betulina* essential oils ranged between 1.0-16.0 mg/ml depending on the pathogen tested. The lowest MIC value was noted against *S. pyogenes* with noteworthy activity (MIC value of 1.0 mg/ml). Other noteworthy activity obtained was against *S. pneumoniae* and *M. smegmatis* (MIC values of 2.0 mg/ml). Some antimicrobial activity was obtained against *C. neoformans*, giving a MIC value of 6.0 mg/ml. Very low antibacterial activity was obtained against *K. pneumoniae*, *M. catarrhalis*, *E. faecalis* and *S. agalactiae* (MIC values ≥8.0 mg/ml).

The antimicrobial activity of the essential oil of buchu was previously studied using the agar diffusion method (Lis-Balchin et al., 2001). The pathogens tested included *E. coli*, *Enterococcus hirae*, *P. aeruginosa*, *Saccharamyces cerevisiae* and *S. aureus*. Very poor to no antimicrobial activity was obtained against the pathogens.

In another study, the antimicrobial activity of the essential oil of *A. betulina* was tested using the MIC micro-titre plate method (Moolla, 2006; Viljoen et al., 2006a). Essential oils tested were from Landmeterskop, Middelberg by Moolla, (2006) and a commercial product was

tested by Viljoen et al., (2006a). Some activity was noted against the pathogens tested i.e. *B. cereus*, *C. albicans*, *K. pneumoniae* and *S. aureus* with MIC values from 4.0-32.0 mg/ml. Results obtained against *K. pneumoniae* (MIC value = 8.0 mg/ml) in the current study were congruent with those mentioned in the review by Moolla and Viljoen, (2008).

Table 5.2 MIC and ∑FIC values of essential oils of *A. afra* and *A. betulina*, alone and in combination.

Micro-organism	MIC (mg/ml)			∑FIC	
	A. afra	***A. betulina***	***A. afra* with *A. betulina***	***A. afra* with *A. betulina* (1:1)**	**Interpretation**
K. pneumoniae NCTC 9633	8.0	8.0	8.0	1.0	Additive
M. catarrhalis ATCC 23246	8.7	16.0	8.0	0.7	Additive
E. faecalis ATCC 29212	8.7	8.0	8.0	1.0	Additive
C. neoformans ATCC 90112	3.8	6.0	4.0	0.9	Additive
S. pneumoniae ATCC 49619	3.3	**2.0**	**2.0**	0.8	Additive
S. pyogenes ATCC 8668	**1.7**	**1.0**	**2.0**	1.6	Indifferent
S. agalactiae ATCC 55618	4.8	8.0	4.0	0.7	Additive
M. smegmatis (clinical)	**1.5**	**2.0**	**2.0**	1.2	Indifferent

*Values in bold indicate noteworthy activity.
Shaded area: results previously discussed in Chapter 3 but included here for reference purposes.
Controls excluded for the sake of brevity. Please refer to Chapter 3 for further detail regarding control values.

For the combination of *A. afra* with *A. betulina,* the lowest MIC value with noteworthy activity (2.0 mg/ml) obtained against the pathogens *S. pneumoniae*, *S. pyogenes* and *M. smegmatis*. Against *C. neoformans* and *S. agalactiae* some antimicrobial activity was noted (MIC values of 4.0 mg/ml). Very low activity was seen against *K. pneumoniae*, *M. catarrhalis* and *E. faecalis* with MIC values of 8.0 mg/ml.

∑FIC values obtained against *K. pneumoniae*, *M. catarrhalis*, *E. faecalis*, *C. neoformans S. pneumoniae* and *S. agalactiae* displayed additive pharmacological interactions. Indifferent interactions were noted against *S. pyogenes* and *M. smegmatis*. Importantly, however, the combination of *A. afra* with *A. betulina* essential oil did not illustrate any antagonism.

5.2.2.1.2 Dichloromethane: methanol extracts

The dichloromethane: methanol extracts of *A. betulina* showed some antimicrobial activity (Table 5.3) against *C. neoformans*, *S. pneumoniae*, *S. pyogenes*, *S. agalactiae* and *M. smegmatis* with MIC values ranging from 1.4-4.0 mg/ml. Moderate inhibition was noted against *S. pneumoniae* (MIC value of 1.4 mg/ml), *S. pyogenes* (1.5 mg/ml) and *M. smegmatis* (1.5 mg/ml). Very poor activity (MIC value of 16.0 mg/ml) was obtained against *M. catarrhalis* while no activity was seen against *K. pneumoniae* and *E. faecalis* at the highest concentration tested. *A. afra* independently showed some antimicrobial activity as noted in Chapter 3.

Moolla et al. (2007) explored the biological activity of the non-volatile fraction of *Agathosma* species. The dichloromethane: methanol extracts of *A. betulina* from Landmeterskop, Middelberg, were tested using the MIC micro-titre plate method against *B. cereus*, *S. aureus*, *K. pneumoniae* and *C. albicans*. The MIC values obtained (2.0-4.0 mg/ml) showed the presence of some activity. The MIC value obtained against *K. pneumoniae* was 4.0 mg/ml.

Sandasi (2008) investigated the antimicrobial activity of the essential oils, dichloromethane: methanol and the methanol extracts of *A. betulina*. MIC micro-titre plate assays were conducted on seven micro-organisms i.e. *L. monocytogenes*, *P. aeruginosa, C. albicans, E. coli, P. vulgaris S. aureus and E. faecalis.* The greatest activity was seen with the dichloromethane: methanol extracts with MIC values from 3.0-6.0 mg/ml. these results correlate with the findings reported here. In the current study, the best result was obtained in the combination of *A. afra* with *A. betulina* against *S. pneumoniae* with a MIC value of 0.6 mg/ml. This demonstrates that the combination of *A. afra* with *A. betulina* is far superior to when used independently for the treatment of microbial infections.

Table 5.3 MIC and ∑FIC values of dichloromethane: methanol extracts of *A. afra* and *A. betulina*, alone and in combination.

Micro-organism	MIC (mg/ml)			∑FIC	
	A. afra	***A. betulina***	***A. afra* with *A. betulina***	***A. afra* with *A. betulina* (1:1)**	**Interpretation**
K. pneumoniae NCTC 9633	6.3	≥16.0	10.3	ND[1]	Additive/indifferent[2]
M. catarrhalis ATCC 23246	4.7	16.0	2.0	**0.3**	**Synergy**
E. faecalis ATCC 29212	6.3	≥16.0	8.0	ND[1]	Additive/indifferent[2]
C. neoformans ATCC 90112	1.2	4.0	1.3	0.7	Additive
S. pneumoniae ATCC 49619	**0.5**	1.4	**0.6**	0.8	Additive
S. pyogenes ATCC 8668	1.1	1.5	1.3	1.0	Additive
S. agalactiae ATCC 55618	3.0	2.5	2.0	0.7	Additive
M. smegmatis (clinical)	1.7	1.5	1.3	0.8	Additive

*Values in bold indicate noteworthy activity.
ND[1] No ∑FIC value could be calculated as no MIC end point for *A. betulina* was obtained at the highest concentration tested.
[2] Tentative interpretation according to MIC data.
Shaded area: results previously discussed in Chapter 3 but included here for reference purposes.
Controls excluded for the sake of brevity. Please refer to Chapter 3 for further detail regarding control values.

The combination of *A. afra* with *A. betulina* showed noteworthy antimicrobial activity against *S. pneumoniae* with an MIC value of 0.6 mg/ml. Some antimicrobial activity was seen against *M. catarrhalis*, *C. neoformans*, *S. pyogenes*, *S. agalactiae* and *M. smegmatis* with moderate inhibition against *C. neoformans*, *S. pyogenes* and *M. smegmatis* (MIC values of 1.3-2.0 mg/ml). Poor activity was observed for *K. pneumoniae* and *E. faecalis*. A very interesting synergistic reaction to the combination against *M. catarrhalis* occurred wherein a ∑FIC value of 0.3 was noted.

∑FIC values could not be calculated for the interactions against *K. pneumoniae* and *E. faecalis* as no end point for *A. betulina* was obtained. However, a tentative interpretation of

the interaction is given. The MIC results of the combination compared to the individual plant extracts against *K. pneumoniae* and *E. faecalis* showed that the interaction is probably additive or indifferent. ∑FIC values calculated against *C. neoformans*, *S. pneumoniae*, *S. pyogenes*, *S. agalactiae* and *M. smegmatis* gave additive interactions for the combination. The antimicrobial activity of the combination was mostly additive indicating a positive rationale for the use with *A. afra* for the treatment of respiratory related diseases.

5.2.2.1.3 Aqueous extracts

The aqueous extracts (Table 5.4) of *A. betulina* were not active against any the pathogens at the highest concentration tested.

Table 5.4 MIC and ∑FIC values of aqueous extracts of *A. afra* and *A. betulina*, alone and in combination.

Micro-organism	**MIC (mg/ml)**			**∑FIC**	
	A. afra	***A. betulina***	***A. afra* with *A. betulina***	***A. afra* with *A. betulina* (1:1)**	**Interpretation**
K. pneumoniae NCTC 9633	12.0	≥16.0	8.0	ND[1]	Additive/indifferent[2]
M. catarrhalis ATCC 23246	8.0	≥16.0	≥16.0	ND[1]	Indifferent/antagonistic[2]
E. faecalis ATCC 29212	8.0	≥16.0	8.0	ND[1]	Additive/indifferent[2]
C. neoformans ATCC 90112	4.0	≥16.0	5.0	ND[1]	Additive/indifferent[2]
S. pneumoniae ATCC 49619	8.0	≥16.0	≥16.0	ND[1]	Indifferent/antagonistic[2]
S. pyogenes ATCC 8668	5.3	≥16.0	≥16.0	ND[1]	Indifferent/antagonistic[2]
S. agalactiae ATCC 55618	1.7	≥16.0	≥16.0	ND[1]	Indifferent/antagonistic[2]
M. smegmatis (clinical)	8.0	≥16.0	≥16.0	ND[1]	Indifferent/antagonistic[2]

*Values in bold indicate noteworthy activity.
ND[1] No ∑FIC value could be calculated as no MIC end point for *A. betulina* was obtained at the highest concentration tested.
[2] Tentative interpretation according to MIC data.
Shaded area: results previously discussed in Chapter 3 but included here for reference purposes.
Controls excluded for the sake of brevity. Please refer to Chapter 3 for further detail regarding control values.

Some poor activity was noted for the combination of *A. afra* with *A. betulina* aqueous extracts against *K. pneumoniae*, *E. faecalis* and *C. neoformans* (MIC values between 5.0-8.0 mg/ml). Due to the lack of susceptibility of any of the selected pathogens to the extracts of *A. betulina*, ∑FIC values could not be calculated. Tentative interpretations of the interactions based on the comparison of the MIC values were made. Additive/indifferent interactions were assumed for the combination against *K. pneumoniae*, *E. faecalis* and *C. neoformans*. Against *M. catarrhalis*, *S. pneumoniae S. pyogenes*, *S. agalactiae* and *M. smegmatis*, the interaction was interpreted as indifferent/antagonistic.

5.2.2.2 Isobologram interpretation

The isobolograms of the interactions of *A. afra* with *A. betulina* essential oils against *M. catarrhalis* (Figure 5.3) showed additive interactions for most of the ratios tested. Two ratios showed indifference i.e. 8:2 and 7:3 (Figure 5.3, a) where *A. afra* is in the majority. The dichloromethane: methanol extracts showed activity wherein three ratios were synergistic, four were additive and two of them indifferent. The ratios showing indifference are 2:8 and 1:9, wherein *A. betulina* is in the majority. The synergistic interactions occur at the ratios 3:7, 4:6 and 1:1 (Figure 5.3, b). The ∑FIC interpretation data correlates with the 1:1 interaction where a FIC value of 0.3 was noted (Table 5.3).

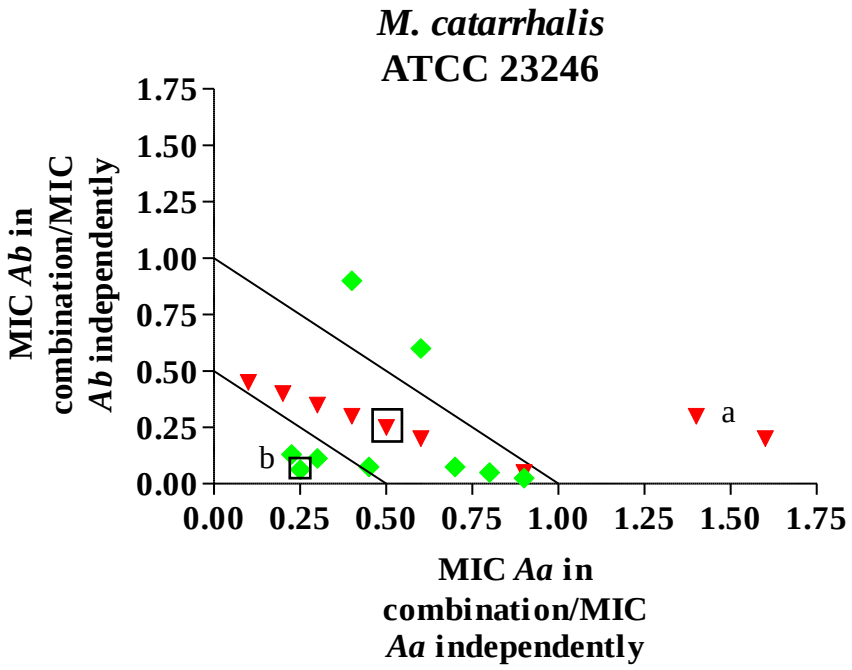


Figure 5.3 Isobologram of the combination of *A. afra* and *A. betulina* essential oils (▼) and the dichloromethane: methanol extracts (■) against *M. catarrhalis* (a – ratios 8:2 and 7:3; b – ratios 3:7, 4:6 and 1:1); □ → 1:1 combination.

The isobologram of the combination of *A. afra* with *A. betulina* against the pathogen *K. pneumoniae* showed additive interactions with all the ratios of the essential oil combination tested (Figure 5.4).

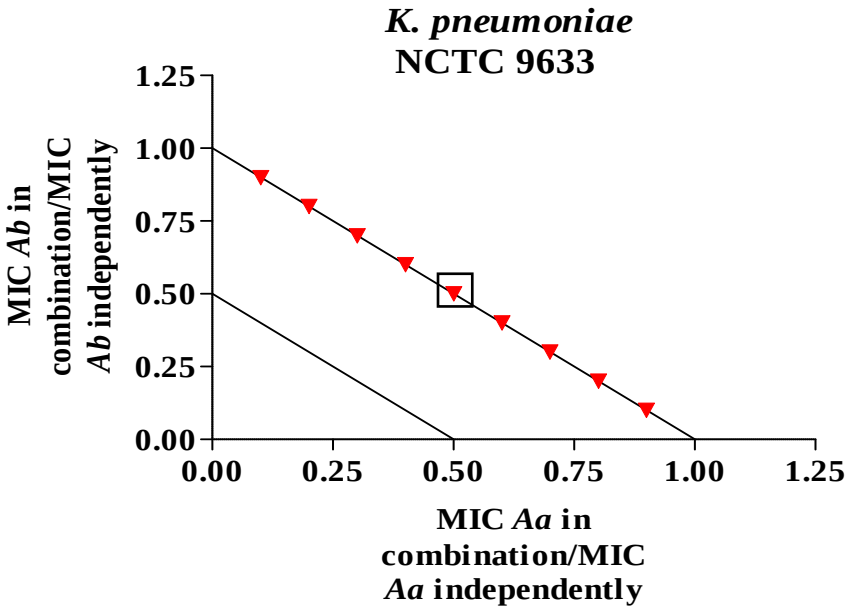


Figure 5.4 Isobologram of the combination of *A. afra* and *A. betulina* essential oils (▼) against *K. pneumoniae*; □→ 1:1 combination.

Figure 5.5 depicts the interactions of *A. afra* with *A. betulina* against *E. faecalis*. The essential oils show additive interactions for all the ratios tested with one point (9:1) showing indifference (Figure 5.5, c).

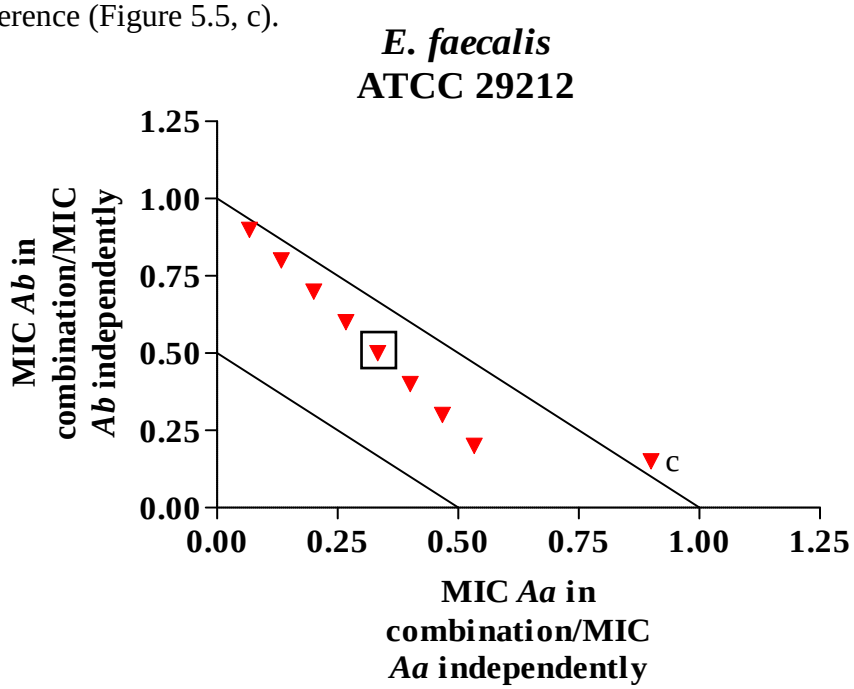


Figure 5.5 Isobologram of the combination of *A. afra* and *A. betulina* essential oils (▼) against *E. faecalis* (c – ratio 9:1); □→ 1:1 combination.

The pathogen *C. neoformans*, tested for antimicrobial susceptibility to various combinations of *A. afra* and *A. betulina* essential oils are illustrated in Figure 5.6.

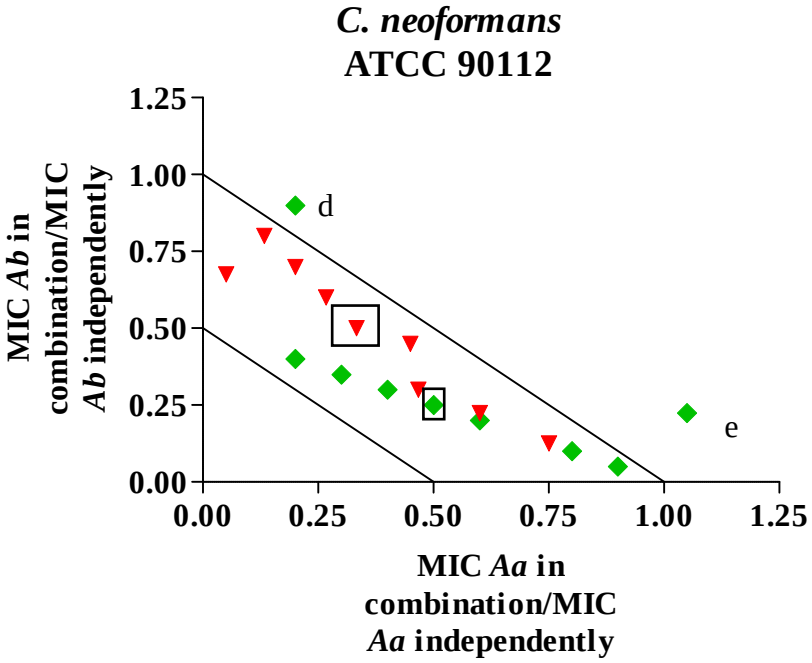


Figure 5.6 Isobologram of the combination of *A. afra* and *A. betulina* essential oils (▼) and dichloromethane: methanol extracts (■) against *C. neoformans* (d – ratio 7:3, e – ratio 1:9); □ → 1:1 combination.

The combination proved to give additive interactions for all ratios tested. The dichloromethane: methanol extracts were additive in this combination for most of the ratios tested with the exception of two (7:3 and 1:9). These two points (Figure 5.6, d and e) proved to be indifferent in their interactions against *C. neoformans*.

The interpretation of the interactions of the 1:1 combination of *A. afra* with *A. betulina* essential oils against *M. catarrhalis*, *K. pneumoniae*, *E. faecalis* and *C. neoformans* showed congruency between the ∑FIC determination and the isobologram method. The combination was also congruent for the dichloromethane: methanol extracts against *M. catarrhalis* and *C. neoformans*.

The dichloromethane: methanol extracts against *K. pneumoniae* and *E. faecalis* did not yield an end point MIC value for *A. betulina*, thus isobolograms could not be drawn. The MIC values of the combination of different ratios of the dichloromethane: methanol extracts of *A. afra* with *A. betulina* are shown in Figure 5.7.

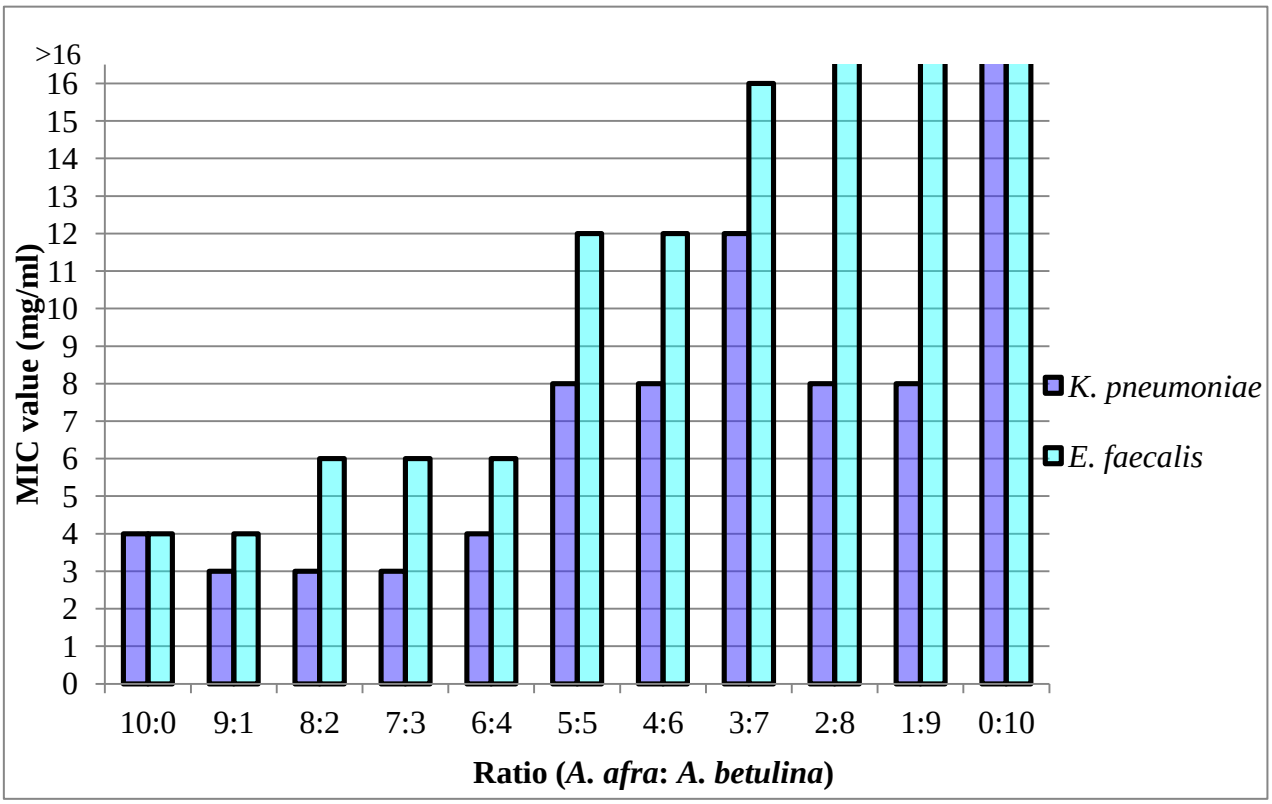


Figure 5.7 MIC values of the different ratios of the combination of *A. afra* with *A. betulina* dichloromethane: methanol extracts against *K. pneumoniae* and *E. faecalis.*

The graph depicts lower MIC values for ratios 9:1, 8:2 and 7:3 against *K. pneumoniae*. These MIC values (3.0 mg/ml) occur when *A. afra* is the dominant extract in the combination. These ratios can therefore be interpreted as being synergistic or additive. As the ratio of *A. betulina* increases, so does the MIC value. The MIC values drop again at ratios 2:8 and 1:9 when the ratio of *A. betulina* is higher. Thus at different ratios, the interaction between the combination varies. This can be interpreted as synergy or additivity at very specific and narrow ratios.

The MIC values obtained for the nine ratios of the dichloromethane: methanol extracts of *A. afra* with *A. betulina* tested against *E. faecalis*, showed an increase in MIC value as the ratio of *A. betulina* increases. The graph serves to demonstrate the change in MIC value as the ratio of plant extracts change.

The aqueous extracts of the combination of *A. afra* with *A. betulina* against *M. catarrhalis*, *E. faecalis*, *K. pneumoniae* and *C. neoformans* could not be plotted onto the isobologram, as *A. betulina* had no end point MIC value. The graph constructed using the MIC data for all the

ratios tested are shown (Figure 5.8). The MIC values generally increases as the percentage of *A. betulina* increases in the ratio combination with *A. afra*.

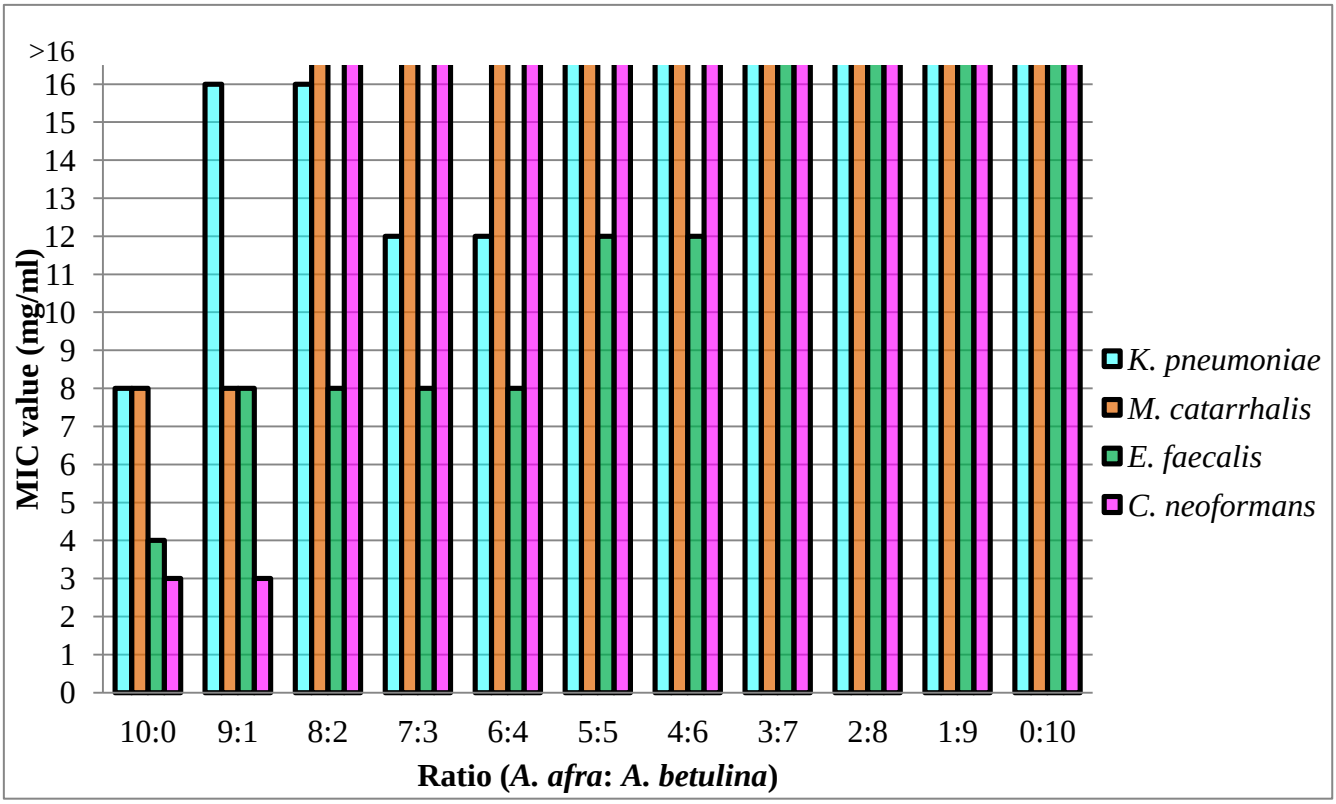


Figure 5.8 The MIC values of the different ratios of the combination of *A. afra* with *A. betulina* aqueous extracts against *K. pneumoniae*, *M. catarrhalis*, *E. faecalis* and *C. neoformans*.

The results from this study have demonstrated that *A. afra* and *A. betulina* may have a better inhibitory effect depending on the dosage forms in which they are tested. This may have selective effects on the interactions of the combination and thus confirms that method of administration is an essential area for future studies in order to better understand the use of combinations in traditional medicine.

Here, the essential oils showed the most additivity in the combination and therefore may validate the use of these plants in an inhaled form. The solvent extracts of the combination showed the only evidence of synergy against *M. catarrhalis*. This is an interesting aspect as the most common use of this combination is an herbal wine.

5.3 Conclusions

- The main constituents found in the essential oil (97.5% of the total composition) of *A. betulina* were limonene (18.9%), menthone (10.0%), isomenthone (31.4%), pseudodiosphenol (7.5%), and diosphenol (8.1%).
- TLC showed the presence of many compounds visible at 254 nm and 365 nm. After derivatization with vanillin-sulphuric acid reagent, many more compounds were made visible.
- The MIC studies revealed the best antimicrobial activity for the essential oil of *A. betulina* was against *S. pyogenes* with a MIC value of 1.0 mg/ml. The best and most noteworthy activity of the combination of the essential oil was against *S. pneumoniae*, *S. pyogenes* and *M. smegmatis* (MIC values of 2.0 mg/ml).
- The dichloromethane: methanol extracts of the combination of *A. afra* with *A. betulina* gave the best activity against *S. pneumoniae* with a MIC value of 0.6 mg/ml.
- *A. betulina* aqueous extracts were inactive at the concentration tested against all eight pathogens. The combination with *A. afra* showed some activity against *K. pneumoniae*, *E. faecalis* and *C. neoformans*.
- Synergy was noted for the combination of the dichloromethane: methanol extracts against *M. catarrhalis* with ∑FIC value of 0.3.
- Synergy was noted for three ratios in the isobologram of the dichloromethane: methanol extracts against *M. catarrhalis.*
- The predominantly additive interactions of the essential oils and the synergy of the dichloromethane: methanol extracts against *M. catarrhalis* observed in the combination of *A. afra* with *A. betulina* give credibility and provides preliminary evidence to support the traditional uses in the treatment of respiratory tract infections.

Chapter 6

The antimicrobial efficacy of *Artemisia afra* Jacq. Ex Willd and *Eucalyptus globulus* Labill. In combination

6.1 Introduction

Although *E. globulus* is not indigenous to South Africa, this medicinal plant is used extensively in African traditional herbal medicine. The combination of *A. afra* with *E. globulus* is one that is used frequently in the treatment of flu-like symptoms and chest-related complaints. The combination of the leaf of *A. afra* and the leaf of *E. globulus* has been said to be used either externally or internally as an influenza remedy (Smith, 1895; Watt and Breyer-Brandwijk, 1962). Additionally a decoction of the combination is used for the common cold or catarrh (Smith, 1895). The steam from a strong decoction of the leaves of the combination of these two plants is said to relieve immediate symptoms (Smith, 1895).

Thus, it is important to include in the evaluation of *A. afra* plant combinations. It is not only commonly used by traditional healers but it also demonstrates the versatility of the traditional healers and their practices to incorporate a variety of medicinal plants into their treatment regimen.

6.2 Results and discussion

6.2.1 Chromatographic techniques

6.2.1.1 Essential oil composition of *E. globulus*

Analysis of the essential oils constituents of *E. globulus* revealed 22 compounds (Table 6.1) by comparison of mass spectral data and retention times of authentic compounds. The main compound found was 1,8-cineole making up 63% of the total composition of the essential oil (96.4%). Other major compounds identified were α-pinene (16.7%), limonene (3.6%) and ρ-cymene (2.7%).

The essential oil of *E. globulus* has been well characterized (Batista-Pereira et al., 2006; Mayaud et al., 2008; George et al., 2009; Tohidpour et al., 2010). Mayaud et al. (2008) performed GC-MS analysis on the essential oil of *E. globulus* supplied by Pranarôm

(International S.A., Ghislenghein, Belgium) and found the main constituents to be 1,8-cineole (80.4%), limonene (7.47%), α, β and γ-terpinene (3.7%) and ρ-cymene (2.5%).

Table 6.1 Essential oil constituents of *E. globulus.*

RRI*	Compounds	Area (%)
1016	**α-Pinene**	**16.7**
1104	β-Pinene	0.3
1193	**Limonene**	**3.6**
1202	**1,8-Cineole**	**63.0**
1270	***p*-Cymene**	**2.7**
1436	*p*-Cymenene	0.1
1441	*trans*-Linalool oxide	0.1
1471	*cis*-Linalool oxide	0.1
1521	Camphor	0.1
1546	Linalool	0.3
1573	Pinocarvone	0.6
1583	Fenchol	0.1
1590	Calarene	0.1
1602	Terpinen-4-ol	0.6
1604	Aromadendrene	1.2
1647	*allo* Aromadendrene	0.2
1662	*trans* Pinocarveol	1.1
1701	α-Terpineol	1.6
1709	Borneol	0.2
1876	*cis* Carveol	0.2
2031	*epi*-Globulol	0.7
2094	Globulol + cubenol	2.8
Total		**96.4**

*RRI: Relative retention indices calculated against *n*-alkanes.
% calculated from TIC data.

George et al. (2009) studied the chemical variation of *E. globulus* from China. The main constituents found were 1,8-cineole (81.9%), D-limonene (7.6%), α-pinene (3.3%) and benzene (3.4%). Previously Cimanga et al. (2002) studied the essential oil composition of *E. globulus* and found less of the 1,8-cineole (44.3%) than George et al. (2009). It is very common to find variation in the chemical composition of an essential oil between different varieties or within a plant (Moreno et al., 2007; George et al., 2009). Tohidpour et al. (2010) found the major constituents of the essential oil of *E. globulus* from Iran to be eucalyptol (47.2%), as the major constituent and most characteristic component of *E. globulus*, (+) spathulenol (18.1%) and α-pinene (9.6%). The percentage of 1,8-cineole found in this study (Table 6.1) was not as much as that of Mayaud et al. (2008) and George et al. (2009), but neither was it as low as that found by Tohidpour et al. (2010). The results of the chemical

composition of *E. globulus* essential oil found in the current study are consistent with the percentage of 1,8-cineole found by George et al. (2009) where the main compound found was 1,8-cineole at 63.0% of the total composition of the essential oil.

6.2.1.2 Thin layer chromatography

The presence of major compounds is detected using TLC (Ahmad and Beg, 2001). Compounds in *E. globulus* that are responsible for activity are alkaloids, phenols and tannins, as described by Ahmad and Beg (2001).

The essential oil (Figure 6.1) of *E. globulus* showed two compounds at 254 nm. These were at R_f values of 0.45 and 0.5. No fluorescent compounds were visible under 365 nm. After derivatization, many compounds became visible. Compound at R_f values of 0.9, 0.25, 0.35 and 0.4 were some that were seen. Once again, the TLC chromatograms showed the complexity of the essential oils alone and the increased complexity in combination.

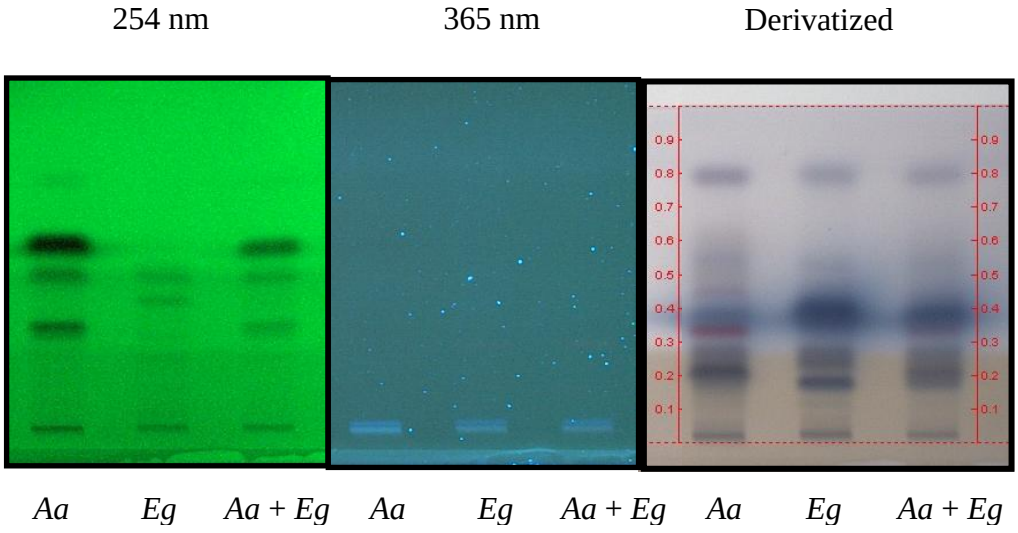


Figure 6.1 TLC of the essential oils of *A. afra* and *E. globulus* individually and in combination at 254 nm, 365 nm and visualized with vanillin-sulphuric acid reagent. *Aa – A. afra*; *Eg – E. globulus*; *Aa + Eg – A. afra* with *E. globulus*.

The TLC chromatograms of the dichloromethane: methanol extracts are depicted in Figure 6.2 Good separation is seen for *E. globulus* individually and in combination with *A. afra*. At 254 nm, many compounds between R_f value 0.35 and 0.55 as well as between R_f 0.8 and 0.9 were seen. Two highly fluorescent red compounds are seen in *E. globulus* at 365 nm at R_f = 0.65 and 0.9 respectively.

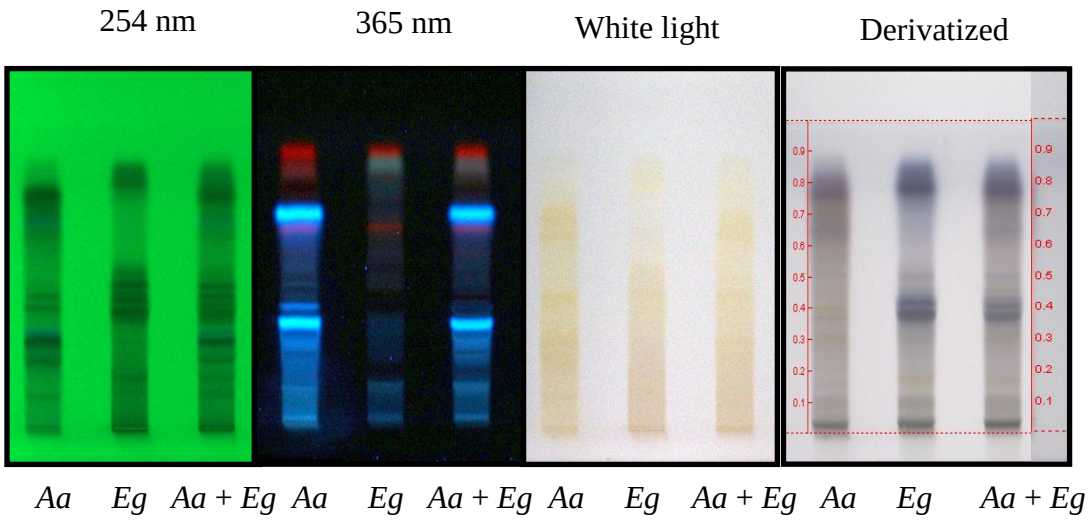


Figure 6.2 TLC of the dichloromethane: methanol extracts of *A. afra* and *E. globulus* individually and in combination at 254 nm, 365 nm, visualized under white light and visualized with vanillin-sulphuric acid reagent. *Aa – A. afra*; *Eg – E.globulus*; *Aa + Eg – A. afra* with *E. globulus*.

The aqueous extracts of *E. globulus* and its combination with *A. afra* are shown in Figure 6.3. At 254 nm, few compounds are visible. Two compounds in *E. globulus* at R_f values of 0.55 and 0.6 are seen at 365 nm, compounds were not visible. Under white light, the separation of compounds showed up mostly yellow.

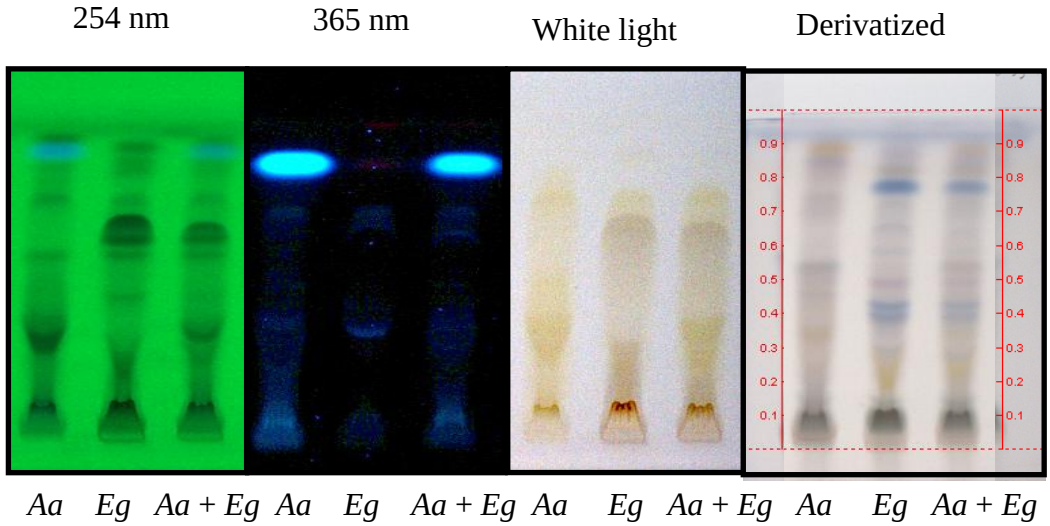


Figure 6.3 TLC of the aqueous extracts of *A. afra* and *E. globulus* individually and in combination at 254 nm, 365 nm, visualized under white light and visualized with vanillin-sulphuric acid reagent. *Aa – A. afra*; *Eg – E. globulus*; *Aa + Eg – A. afra* with *E. globulus*.

Compounds were much more visible after derivatization for *E. globulus* and the combination (Figure 6.3). Blue compounds were seen for *E. globulus* and in the combination at R_f values of 0.39, 0.41 and 0.76.

6.2.2 Antimicrobial analysis

6.2.2.1 MIC assays and FIC determination

6.2.2.1.1 Essential oils

Antimicrobial studies of *E. globulus* essential oils (Table 6.2) showed diverse results. *E. globulus* against *K. pneumoniae*, *M. catarrhalis* and *E. faecalis* showed very poor antimicrobial activity with MIC values of 8.0 mg/ml. Some antimicrobial activity was obtained against *S. pneumoniae* (MIC value = 2.7 mg/ml) and *S. agalactiae* (5.3 mg/ml). Noteworthy activity for the essential oil *E. globulus* was obtained against *S. pyogenes* (MIC value = 2.0 mg/ml) and *M. smegmatis* (MIC value = 2.0 mg/ml). The best activity was noted against *C. neoformans* yielding an MIC value of 0.6 mg/ml,

Cimanga et al. (2002) tested the antimicrobial activity of *E. globulus* essential oil using the disc diffusion method. The pathogens included *E. coli*, *B. subtilis*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus* and *S. flexneri*. The activity against two *K. pneumoniae* strains were interpreted as very active and against *P. aeruginosa* the oil was moderately active.

The antimicrobial properties of the essential oil of *E. globulus* against *S. aureus* and *E. coli* were confirmed using the aromatogramme, microastmosphere and germs in suspension methods (Ghalem and Mohamed, 2008a). In another study on the antimicrobial activity of the essential oil of *E. globulus*, the agar dilution method was used to calculate mean MIC percentages (Mayaud et al., 2008). Activity was assessed on a number of micro-organisms including *K. pneumoniae* (mean MIC = 10.0%) and *E. faecalis* (mean MIC = 5.83 ±SD of 1.86%). Twelve *Streptococci* spp were also evaluated for their potential susceptibility (mean MIC = 2.78±SD of 2.08%). Good reproducibility and results were found in terms of MIC's.

The antimicrobial activity of an essential oil is linked to the chemical composition of the oil i.e. the presence of alcohols, phenols, terpenes and ketones (Iserin, 1997; Inouye et al., 2001; Cimanga et al., 2002; Ghalem and Mohamed, 2008a, b).

Table 6.2 MIC and ∑FIC values the essential oils of *A. afra* and *E. globulus* alone and in combination.

Micro-organism	MIC (mg/ml)			∑FIC	
	A. afra	***E. globulus***	***A. afra* with *E. globulus***	***A. afra* with *E. globulus* (1:1)**	**Interpretation**
K. pneumoniae NCTC 9633	8.0	8.0	8.0	1.0	Additive
M. catarrhalis ATCC 23246	8.7	8.0	8.0	1.0	Additive
E. faecalis ATCC 29212	8.7	8.0	8.0	1.0	Additive
C. neoformans ATCC 90112	3.8	**0.6**	**0.8**	0.8	Additive
S. pneumoniae ATCC 49619	3.3	2.7	3.0	1.0	Additive
S. pyogenes ATCC 8668	**1.7**	**2.0**	**1.5**	0.8	Additive
S. agalactiae ATCC 55618	4.8	5.3	4.0	0.8	Additive
M. smegmatis (clinical)	**1.5**	**2.0**	**1.3**	0.7	Additive

*Values in bold indicate noteworthy activity.
Shaded area: results previously discussed in Chapter 3 but included here for reference purposes.
Controls excluded for the sake of brevity. Please refer to Chapter 3 for further detail regarding control values.

Recently Tohidpour et al. (2010) tested the essential oil of *E. globulus* using the disk diffusion and the agar diffusion methods. Zones of inhibition and MIC values were calculated for *B. cereus, E. coli*, *K. pneumoniae,* Methicillin resistant *S. aureus* (MRSA) and *S. aureus. E. coli* was the most sensitive pathogen tested with an inhibition zone of 8 mm and a MIC value of 51.36 µg/ml. *K. pneumoniae* yielded an inhibition zone of 8 mm and the MIC value obtained was 68.48 µg/ml. Some variation against *K. pneumoniae* is evident between this current study and that undertaken by Tohidpour et al. (2010). This is possibly due to the different methods of evaluation of antimicrobial activity that were used.

In the combination of *A. afra* and *E. globulus* essential oils, the same trend of antimicrobial activity as that of *E. globulus* alone was noticed. Very poor activity was seen against *K. pneumoniae*, *M. catarrhalis* and *E. faecalis* (MIC values of 8.0 mg/ml). Some antimicrobial

activity was seen against *S. pneumoniae* (MIC value = 3.0 mg/ml) and *S. agalactiae* (4.0 mg/ml). Noteworthy activity was obtained against *C. neoformans* yielding the best MIC value of the combination of 0.8 mg/ml, *S. pyogenes* (MIC value = 1.5 mg/ml) and *M. smegmatis* (MIC value = 1.3 mg/ml).

The ∑FIC values calculated (Table 6.2) for the combination of *A. afra* with *E. globulus* essential oil against all the pathogens tested, yielded additive pharmacological interactions.

6.2.2.1.2 Dichloromethane: methanol extracts

The dichloromethane: methanol extract of *E. globulus* against all the pathogens tested, showed antimicrobial activity with MIC values between 0.01 - 3.0 mg/ml (Table 6.3). Some activity was noted against *K. pneumoniae* (MIC value = 3.0 mg/ml). Moderate inhibitory antimicrobial activity was noted against *S. agalactiae* and *M. smegmatis* with MIC values of 1.0 and 1.5 mg/ml respectively. Against *M. catarrhalis* and *E. faecalis, C. neoformans, S. pneumoniae* and *S. pyogenes* noteworthy activity was obtained for *E. globulus. M. catarrhalis* and *E. faecalis* exhibited very strong inhibitory activity with MIC values of 0.01 mg/ml and 0.02 mg/ml respectively. Moderate inhibition was noted with *C. neoformans* (MIC value of 0.5 mg/ml) and *S. pyogenes* (MIC value of 0.6 mg/ml). Strong inhibition was noted against *S. pneumoniae* where a MIC value of 0.2 mg/ml was obtained.

The antimicrobial activity of *E. globulus* ethanol extracts against *S. pyogenes* is supported by a study by Caceres et al. (1991). The study found that *E. globulus* clearly inhibited the *in vitro* growth of *S. aureus* as well as *S. pyogenes*. Takahashi et al. (2004) tested the antimicrobial activity of *E. globulus* dichloromethane: methanol extracts on nine micro-organisms. The MIC value obtained against *E. faecalis* was 31 mg/l (0.031 mg/ml) which was consistent with that found in this study where a MIC value of 0.02 mg/ml was obtained. Khan et al. (2009a) recently studied the ethanol extracts of *E. globulus* against a number of pathogens including *E. faecalis* and two strains of *K. pneumoniae*. The extract was found to be inactive at the concentration tested against both strains of *K. pneumoniae*. However, against *E. faecalis* an MIC value of 3130 µg/ml (3.13 mg/ml) was found. This activity noted was not as good as that reported in this study. This variation could be due to the type of extract tested was different or the strain of the organism was not the same.

Table 6.3 MIC and ∑FIC values of the dichloromethane: methanol extracts of *A. afra* and *E. globulus* alone and in combination.

Micro-organism	MIC (mg/ml)			∑FIC	
	A. afra	***E. globulus***	***A. afra* with *E. globulus***	***A. afra* with *E. globulus* (1:1)**	**Interpretation**
K. pneumoniae NCTC 9633	6.3	3.0	2.0	**0.5**	**Synergy**
M. catarrhalis ATCC 23246	4.7	**0.01**	1.1	5.8	Antagonism
E. faecalis ATCC 29212	6.3	**0.02**	2.1	7.6	Antagonism
C. neoformans ATCC 90112	1.2	**0.5**	**0.8**	1.1	Indifferent
S. pneumoniae ATCC 49619	**0.5**	**0.2**	**0.3**	0.9	Additive
S. pyogenes ATCC 8668	1.1	**0.6**	**0.3**	**0.3**	**Synergy**
S. agalactiae ATCC 55618	3.0	1.0	1.0	0.7	Additive
M. smegmatis (clinical)	1.7	1.5	**0.5**	**0.3**	**Synergy**

*Values in bold indicate noteworthy activity.
Shaded area: results previously discussed in Chapter 3 but included here for reference purposes.
Controls excluded for the sake of brevity. Please refer to Chapter 3 for further detail regarding control values.

In the combination of *A. afra* with *E. globulus* dichloromethane: methanol extracts, MIC values were all below 3.0 mg/ml. Some antimicrobial activity was seen against *K. pneumoniae* and *E. faecalis* while moderate inhibition was noted against *M. catarrhalis* and *S. agalactiae*. Of particular interest are the MIC values against *C. neoformans*, *S. pneumoniae*, *S. pyogenes* and *M. smegmatis* with MIC's of 0.8, 0.3, 0.3 and 0.5 mg/ml respectively. These values were interpreted as strong inhibitory activity for the combination of *A. afra* with *E. globulus* dichloromethane: methanol extract.

∑FIC values were calculated and only one of the combinations showed indifferent interactions i.e. against *C. neoformans*. Additive interactions were noted for the pathogens *S. pneumoniae* and *S. agalactiae*. Synergistic interactions were found against *K. pneumoniae* (∑FIC value = 0.5), *S. pyogenes* and *M. smegmatis* with ∑FIC values of 0.3. For the first time

in the study, antagonism was noted and seen against *M. catarrhalis* and *E. faecalis* with very high ∑FIC values (5.8 and 7.6). If one examines the very low MIC values of *E. globulus* independently against *M. catarrhalis* and *E. faecalis* and then examines the combined efficacy, it is clear that when treating infections from these two pathogens it is not advisable to combine with *A. afra*.

Looking at the traditional method of administration of the combination when it is administered through inhalation of the steam from the infusions or decoctions are administered (Hutchings et al., 1996). The alcohol extracts are not cited as being used traditionally this shows that care should be taken when using the combinations as the type of infection is not known to a traditional healer. Proper diagnostic tests should be considered to isolate the pathogen responsible for the infection prior to treatment. This antagonism seen could cause unwanted side effects and toxicity, or the plant extracts could be potentially harmful. Further investigations in this regard are recommended.

6.2.2.1.3 Aqueous extracts

The aqueous extracts of *E. globulus*, as shown in Table 6.4, gave MIC values between 0.8 - 14.0 mg/ml against all the pathogens tested. Very poor activity was noted against *S. pneumoniae* with a MIC value of 14.0 mg/ml. Some antimicrobial activity was seen against *K. pneumoniae, M. catarrhalis, E. faecalis* and *M. smegmatis.* Moderate inhibition was obtained against *S. pyogenes* and *S. agalactiae* and noteworthy antimicrobial activity was seen against *C. neoformans* with a MIC value of 0.8 mg/ml.

In the combination with *A. afra*, antimicrobial activities ranged between 0.4-8.0 mg/ml against the pathogens tested. Moderate inhibition was seen, with an MIC value of 1.0 mg/ml, against *S. agalactiae*. Some activity was observed in combination against pathogens *C. neoformans, S. pneumoniae* and *S. agalactiae*. Noteworthy strong inhibition was obtained against *C. neoformans* with a MIC value of 0.4 mg/ml.

∑FIC values determined revealed no antagonism in the combination against any of the pathogens tested. Synergy was obtained against *C. neoformans* and *S. pneumoniae* (∑FIC values of 0.3 and 0.2). Additive interactions were noted against *K. pneumoniae, M. catarrhalis, S. pyogenes, S. agalactiae* and *M. smegmatis*. Against *E. faecalis*, an indifferent pharmacological interaction was noted.

Table 6.4 MIC and ∑FIC values of the aqueous extracts of *A. afra* and *E. globulus* alone and in combination.

Micro-organism	MIC (mg/ml)			∑FIC	
	A. afra	***E. globulus***	***A. afra* with *E. globulus***	***A. afra* with *E. globulus* (1:1)**	**Interpretation**
K. pneumoniae NCTC 9633	12.0	6.0	8.0	1.0	Additive
M. catarrhalis ATCC 23246	8.0	3.5	4.0	0.8	Additive
E. faecalis ATCC 29212	7.0	2.5	4.0	1.1	Indifferent
C. neoformans ATCC 90112	4.0	**0.8**	**0.4**	**0.3**	**Synergy**
S. pneumoniae ATCC 49619	8.0	14.0	2.0	**0.2**	**Synergy**
S. pyogenes ATCC 8668	5.3	1.3	2.0	0.9	Additive
S. agalactiae ATCC 55618	1.7	1.5	1.0	0.6	Additive
M. smegmatis (clinical)	8.0	4.0	4.0	0.8	Additive

*Values in bold indicate noteworthy activity.
Shaded area: results previously discussed in Chapter 3 but included here for reference purposes.
Controls excluded for the sake of brevity. Please refer to Chapter 3 for further detail regarding control values.

6.2.2.2 Isobologram interpretation

The essential oils (Figure 6.4 and inset) of the combination of *A. afra* with *E. globulus* against *M. catarrhalis* showed additive interactions for all the ratios tested. The dichloromethane: methanol extracts were indifferent at four ratios tested and completely antagonistic for five of them (Figure 6.4). The ratios in which antagonism were noted are at 8:2, 7:3, 6:4, 5:5 and 4:6. *A. afra* and *E. globulus* vary in concentration at these ratios, but the general trend suggests that when the two extracts come closer in concentration to each other (1:1), antagonism is the result. In contrast, the aqueous extracts (Figure 6.4 and inset) were synergistic at all ratios tested except one. The one exception was the 1:1 ratio and it showed an additive interaction. This is interesting to note as the traditional use is the inhalation of the steam of an infusion or a decoction of the combination is taken (Hutchings et al., 1996).

Furthermore, results of aqueous extracts against *M. catarrhalis* demonstrate that 1:1 combinations may not always be the best selection.

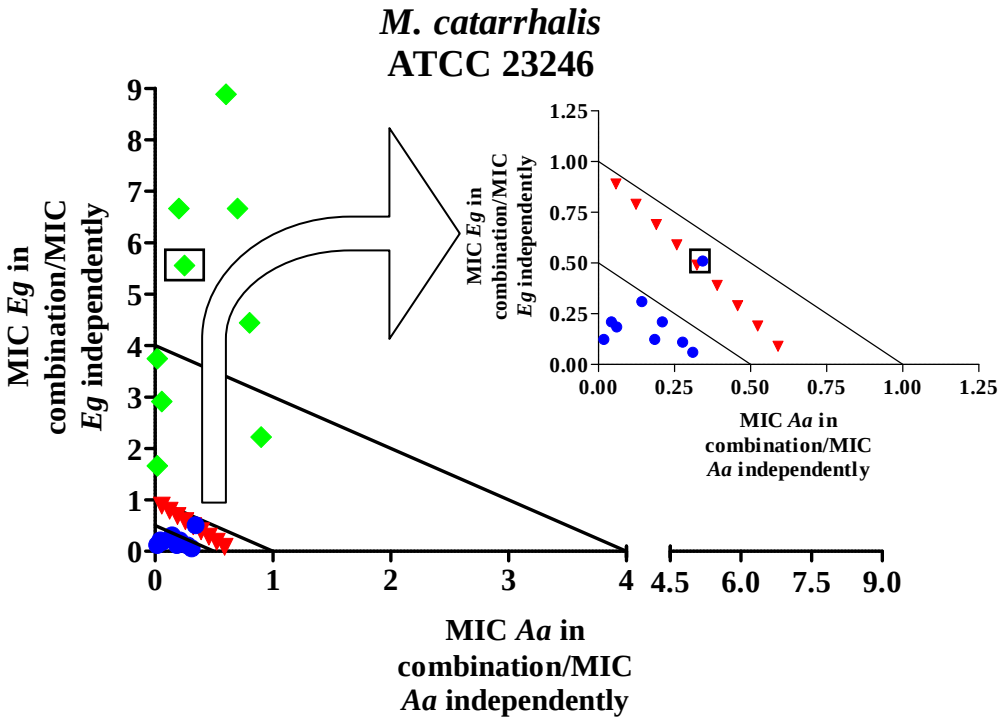


Figure 6.4 Isobologram of the combination of *A. afra* and *E. globulus* essential oils (▼), dichloromethane: methanol extracts (■) and aqueous extracts (●) against *M. catarrhalis*; □ → 1:1 combination.

For *K. pneumoniae* (Figure 6.5), the essential oils of the combination showed additive interactions for all the ratios tested. The dichloromethane: methanol extracts showed additive interactions for most of the ratios. However, two exceptions at ratios 4:6 and 5:1 (Figure 6.5, a) were synergistic. This synergy occurred when *A. afra* and *E. globulus* were at the same ratio and when *E. globulus* was slightly more than *A. afra* (ratio 4:6). The aqueous extracts of the combination of *A. afra* with *E. globulus* showed a mixture of indifferent and additive interactions. Additivity was noted at ratios 6:4, 4:6, 3:7, 2:8 and 1:9 and the 1:1 combination. Indifferent interactions were seen at ratios 9:1, 8:2 and 7:3. A higher concentration of *E. globulus* is better to obtain more synergy in the combination of the aqueous extracts against *K. pneumoniae*.

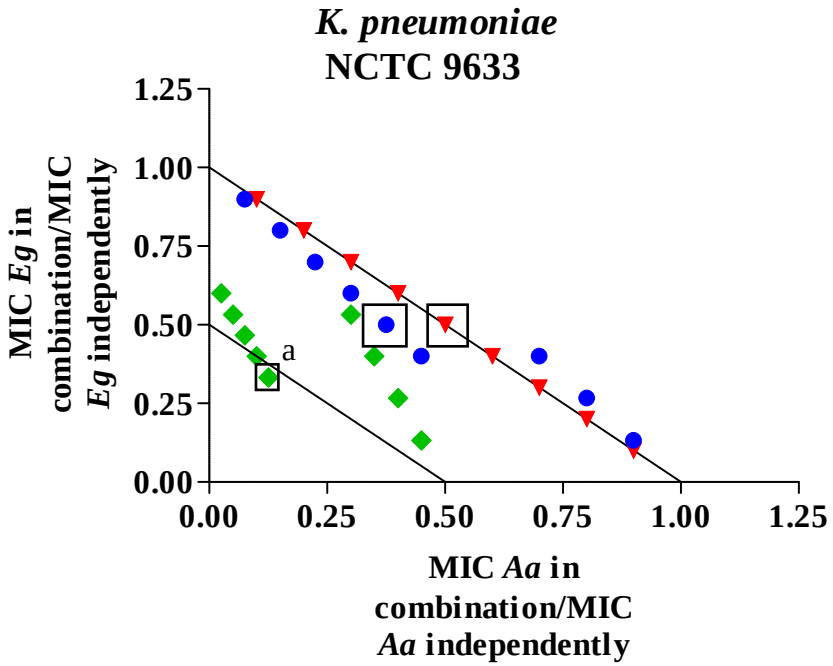


Figure 6.5 Isobologram of the combination of *A. afra* and *E. globulus* essential oils (▼), dichloromethane: methanol extracts (■) and aqueous extracts (●) against *K. pneumoniae* (a - ratios 4:6 and 5:1); □→ 1:1 combination.

In Figure 6.6, the interactions of the combination of *A. afra* with *E. globulus* against *E. faecalis* is shown. The essential oil (Figure 6.6, inset) results show additive interactions for all the ratios tested.

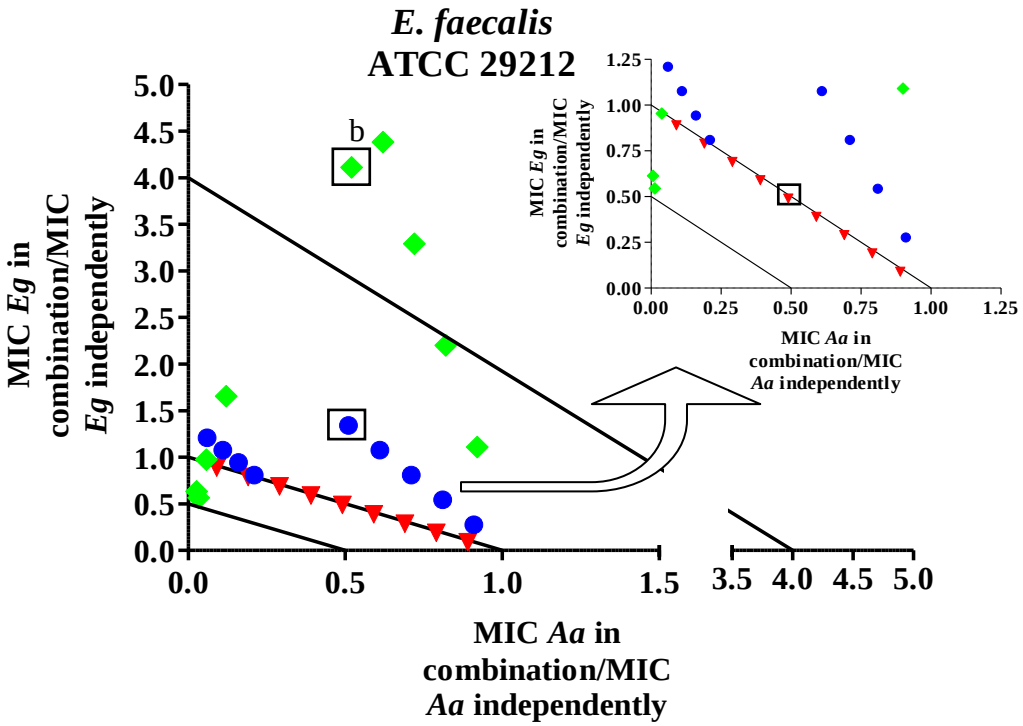


Figure 6.6 Isobologram of the combination of *A. afra* and *E. globulus* essential oils (▼), dichloromethane: methanol extracts (■) and aqueous extracts (●) against *E. faecalis* (b – ratios 6:4 and 1:1); □ → 1:1 combination.

The dichloromethane: methanol extracts (Figure 6.6) showed additive interactions for three ratios (3:7, 2:8 and 1:9), where *E. globulus* is the dominant essential oil in the combination. Four of the ratios were indifferent and two ratios were antagonistic Figure 6.6 shows the antagonistic ratios at (b), which are the 6:4 and the 5:5 combinations of the essential oils. The aqueous extracts showed indifferent interactions for all the ratios tested, except one, which was additive (ratio 4:6).

The isobolographic interactions of the combination against *C. neoformans* (Figure 6.7) show additive interaction for the essential oils at all the ratios tested. The dichloromethane: methanol extracts showed mixed interactions. Ratios 9:1, 8:2, 4:6, 1:9 and 3:7 show additive interactions while ratios 7:3, 6:4, 2:8, and the 1:1 combinations were indifferent. The aqueous extracts displayed very interesting activity with all the ratios to be synergistic.

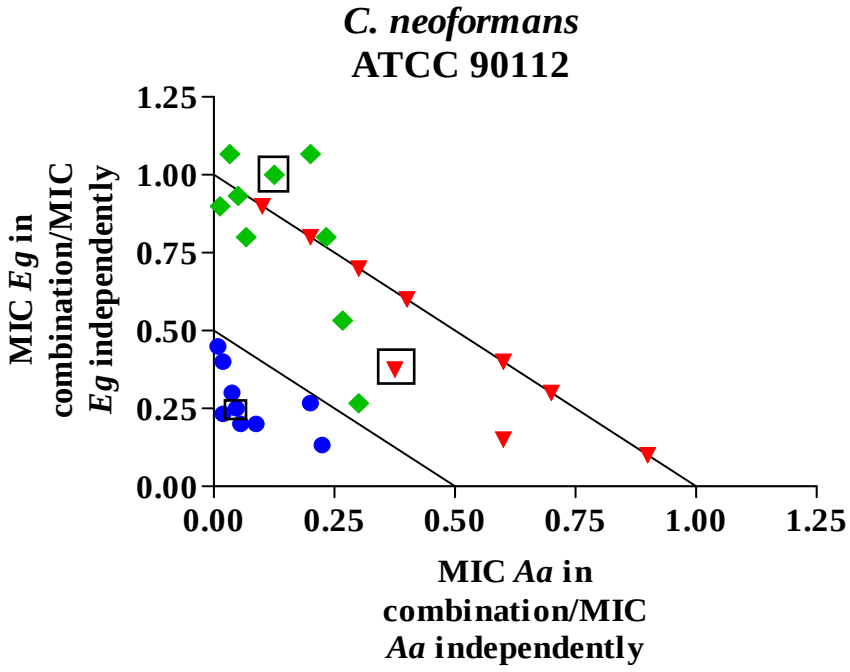


Figure 6.7 Isobologram of the combination of *A. afra* and *E. globulus* essential oils (▼), dichloromethane: methanol extracts (■) and aqueous extracts (●) against *C. neoformans*; □ → 1:1 combination.

The interactions of the 1:1 combination evaluated using the ∑FIC method and the isobologram interaction method were in congruence with each other against *M. catarrhalis* and *E. faecalis*. Variation was noted against *K. pneumoniae* with the dichloromethane: methanol extracts where the ∑FIC value indicated additivity and the isobologram was synergistic. The aqueous extracts against *K. pneumoniae* also expressed some variation where

the ∑FIC interpretation was indifferent and the isobologram interpretation was additive. The essential oil results of the interactions were congruent.

Against *C. neoformans*, the variations occurred in the essential oil 1:1 combination wherein the ∑FIC interpretation was synergistic and the isobologram interpretation was additive. The dichloromethane: methanol extract combination showed additivity when the ∑FIC was calculated and indifference with the isobologram.

The interactions observed for the 1:1 combination against *M. catarrhalis, E. faecalis, K. pneumoniae* and *C. neoformans* using the ∑FIC determination method were in congruence with those observed when using the isobologram method.

The combination of *E. globulus* essential oil with chlorhexidine dicgluconate has been studied by Karpanen et al. (2008). The MIC chequerboard method was used to evaluate the antimicrobial activity against two strains of *S. epidermidis* of the combination, in suspension and in biofilms. Using the ∑FIC determination method, the interactions of the combination in suspension was indifferent. However, when *E. globulus* was combined with chlorhexidine dicgluconate against both strains of *S. epidermidis* growing in biofilms, synergistic activity was obtained.

It is fascinating to note that the interactions of the combination, whether evaluated by MIC, ∑FIC or the isobolograms, showed that the essential oil were mostly additive. In Low et al. (1974) (Hasegawa et al., 2008), the essential oils of *Eucalyptus* spp is used for the treatment of pulmonary infections by inhalation. This is also the case in the combination of *A. afra* with *E. globulus*. The traditional use is cited in Hutchings et al. (1996) wherein it states that the combination is administered by the inhalation of the steam of the infusion. The results obtained in the study show the essential oils exhibit the most consistent activity with predominantly additive interactions against the four pathogens tested. Thus, correlates with the traditional use of the combination by traditional healers.

The dichloromethane: methanol extracts in combination however, were the most antagonistic in their interactions against *M. catarrhalis.* Additivity or indifference was predominant against *E. faecalis, K. pneumoniae* and *C. neoformans*.

The aqueous extracts showed very good activity and if not synergistic interactions the ratios were additive against *M. catarrhalis*, *K. pneumoniae* and *C. neoformans*. Indifference was noted from three ratios against *K. pneumoniae* and against *E. faecalis*. These results are interesting as none of the combinations exhibited antagonism against the pathogens tested. In addition, the literature cites the combination to be taken as decoction (Hutchings et al., 1996). Thus, the traditional method of administration ties in with the results obtained. This serves to validate the use of *A. afra* in combination with *E. globulus* essential oils and aqueous extract to treat respiratory infections.

6.3 Conclusions

- The major compounds present in *E. globulus* were 1,8-cineole (63%) and α-pinene (16.7%), limonene (3.6%) and ρ-cymene (2.7%).
- The essential oil of *E. globulus* showed some antimicrobial activity with the best and noteworthy activity against *C. neoformans* (MIC value of 0.6 mg/ml).
- The dichloromethane: methanol extract of E. *globulus* showed very good antimicrobial activity against, *C. neoformans* (MIC value = 0.5 mg/ml), *S. pneumoniae* (MIC value = 0.19 mg/ml) and *S. pyogenes* (MIC value = 0.56 mg/ml) with the best activity against *E. faecalis* with a MIC value of 0.02 mg/ml and *M. catarrhalis* (MIC value = 0.014 mg/ml).
- In the combination of the dichloromethane: methanol extracts of *A. afra* with *E. globulus*, the best antimicrobial activity was obtained against *S. pneumoniae* (MIC value = 0.25 mg/ml), *S. pyogenes* (MIC value = 0.25 mg/ml) and *M. smegmatis* (MIC value = 0.5 mg/ml).
- The ∑FIC determination method of the dichloromethane: methanol extract combination gave antagonistic interactions against *M. catarrhalis* and *E. faecalis*. Synergy was noted against *K. pneumoniae*, *S. pyogenes* and *M. smegmatis*.
- The best MIC value obtained with the aqueous extract of *E. globulus* was 0.8 mg/ml against *C. neoformans*. The combination with *A. afra* also gave the best MIC value against *C. neoformans* (MIC value of 0.4 mg/ml).
- The combination of the aqueous extracts showed synergistic ∑FIC interactions against *C. neoformans* (0.3) and *S. pneumoniae* (0.2)

- The essential oils of the combination of *A. afra* with *E. globulus* showed additive interactions against *M. catarrhalis*, *K. pneumoniae*, *E. faecalis* and *C. neoformans* when assessing the isobolograms.
- The isobolograms showed antagonistic interactions (five ratios) for the dichloromethane: methanol extract of the combination against *M. catarrhalis*. The aqueous extracts of the combination showed very promising results with most or all ratios showing synergy against *M. catarrhalis* and *C. neoformans*.
- Overall, *C. neoformans* was the most sensitive pathogen to the combination of *A. afra* with *E. globulus* essential oils and aqueous extracts.

Chapter 7

The antimicrobial efficacy of *Artemisia afra* Jacq. ex Willd and *Zanthoxylum capense* (Thunb.) Harv. in combination

7.1 Introduction

The combination of *A. afra* with *Z. capense* has been used by traditional healers for the treatment of head colds and influenza (Watt and Breyer-Brandwijk, 1962; Hutchings et al., 1996). It was a popular remedy in the influenza epidemic in 1918. A decoction and an infusion of the leaves of *Z. capense* is made with *A. afra* and cited as been used in febrile conditions. If it was used as a remedy for influenza since 1918, it follows that the combination be analyzed for its validity in treatment of respiratory illnesses. Even though *Z. capense* is aromatic in nature, no essential oil was obtained through hydro-distillation. The dichloromethane: methanol extracts and the aqueous extracts were thus only studied.

7.2 Results and discussion

7.2.1 Chromatographic techniques

7.2.1.1 Thin layer chromatography

Thin layer chromatography analysis of the dichloromethane: methanol extracts of *Z. capense* revealed at 254 nm (Figure 7.1), that many of the compounds did not migrate up the plate. It is also evident at 365 nm that a blue fluorescent band was visible at the bottom. Many fluorescent red compounds are also visible at 365 nm. In *Z. capense* alone and in the combination with *A. afra*, a dark green band is visible at $R_f = 0.9$. A bright yellow compound at $R_f = 0.8$ is also seen under white light (Figure 7.1). After derivatization, the compounds mentioned are seen as bluish-purple.

Three fluorescent compounds seen at 365 nm are dominant in the combination of *A. afra* with *Z. capense* dichloromethane: methanol extracts. These are shown in Figure 7.1. two compounds are from *A. afra* and extract 1 compound is from *Z. capense* extract.

The aqueous extracts as seen in Figure 7.2, showed a limited number of compounds at 254 nm.

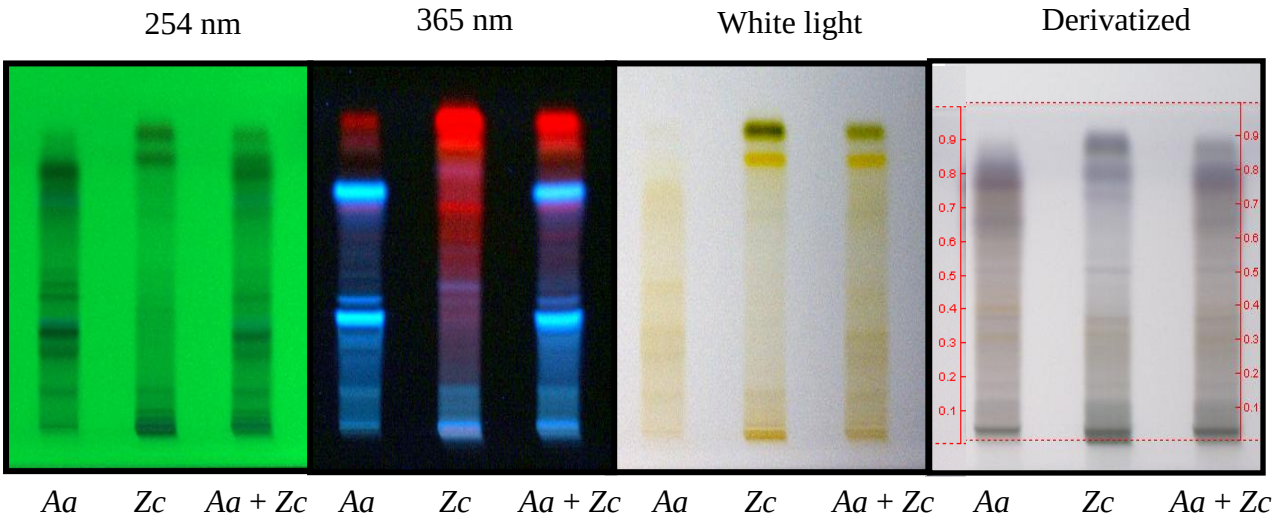


Figure 7.1 *A. afra* dichloromethane: methanol extracts alone and in combination with *Z. capense* visualizing at 254 nm, 365 nm, white light and after derivatization with vanillin-sulphuric acid. *Aa - A. afra*; *Zc - Z.capense*; *Aa + Zc - A. afra* with *Z. capense*.

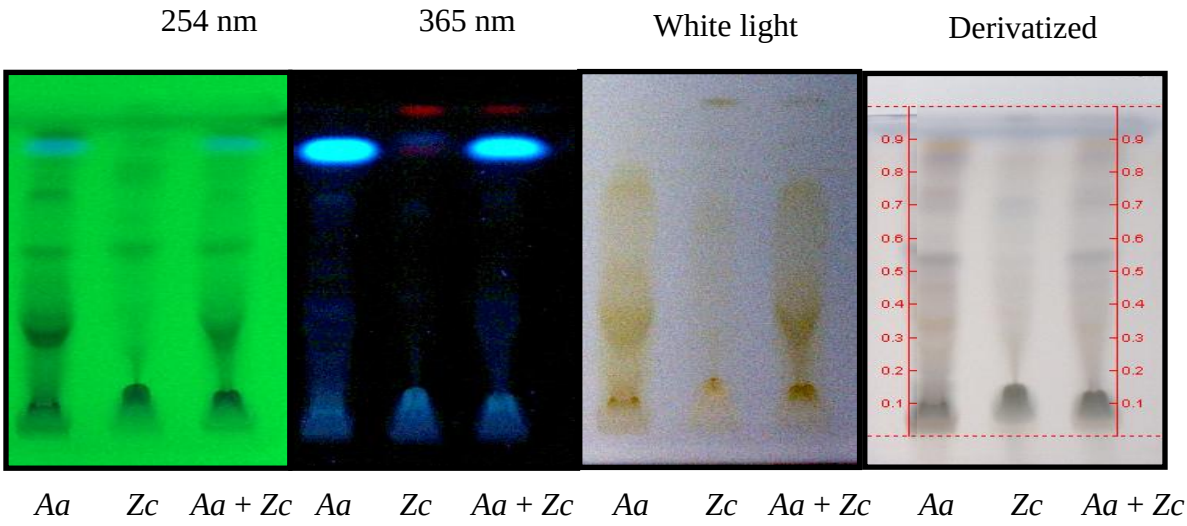


Figure 7.2 *A. afra* aqueous extracts alone and in combination with *Z. capense* visualizing at 254 nm, 365 nm, white light and after derivatization with vanillin-sulphuric acid. *Aa - A. afra; Zc - Z.capense; Aa + Zc - A. afra with Z. capense*.

7.2.2 Antimicrobial analysis

7.2.2.1 MIC assays and FIC determination

7.2.2.1.1 Dichloromethane: methanol extracts

For the dichloromethane: methanol extracts (Table 7.1), moderate inhibitory antimicrobial activity was noted against *E. faecalis* and *S. pneumoniae*. Some antimicrobial activity was noted against *M. catarrhalis*, *K. pneumoniae*, *S. pyogenes* and *S. agalactiae*. Noteworthy

activity with strong inhibition was obtained against *C. neoformans* (MIC value = 0.4 mg/ml) and moderate inhibition against *M. smegmatis* with MIC values of 0.8 mg/ml.

Table 7.1 MIC and ∑FIC values of *A. afra* and *Z. capense* dichloromethane: methanol extracts independently and in combination.

Micro-organism	MIC (mg/ml)			∑FIC	
	A. afra	***Z. capense***	***A. afra* with *Z. capense***	***A. afra* with *Z. capense* (1:1)**	**Interpretation**
K. pneumoniae NCTC 9633	6.3	1.7	2.0	0.8	Additive
M. catarrhalis ATCC 23246	4.7	2.0	2.0	0.7	Additive
E. faecalis ATCC 29212	6.3	1.3	2.0	0.9	Additive
C. neoformans ATCC 90112	1.2	**0.4**	**0.4**	0.7	Additive
S. pneumoniae ATCC 49619	**0.5**	1.5	**0.3**	**0.4**	**Synergy**
S. pyogenes ATCC 8668	1.1	2.0	1.0	0.7	Additive
S. agalactiae ATCC 55618	3.0	4.0	4.0	1.2	Indifference
M. smegmatis (clinical)	1.7	**0.8**	2.0	1.9	Indifference

*Values in bold indicate noteworthy activity.
Shaded area: results previously discussed in Chapter 3 but included here for reference purposes.
Controls excluded for the sake of brevity. Please refer to Chapter 3 for further detail regarding control values.

Antimicrobial activity (moderate inhibition) was noted for the combination of *A. afra* with *Z. capense* dichloromethane: methanol extracts, against *S. pyogenes* with an MIC value of 1.0 mg/ml. Some antimicrobial activity was seen against *K. pneumoniae*, *M. catarrhalis*, *E. faecalis*, *S. agalactiae* and *M. smegmatis*. The lowest MIC values and noteworthy inhibition were obtained against pathogens *C. neoformans* (0.4 mg/ml) and *S. pneumoniae* (0.3 mg/ml)

∑FIC values of the combination of the dichloromethane: methanol extracts of *A. afra* with *Z. capense* demonstrated synergy against *S. pneumoniae* (∑FIC value = 0.4). Additive ∑FIC values were obtained for the combination against *K. pneumoniae, M. catarrhalis, E. faecalis,*

C. neoformans and *S. pyogenes*. Indifferent interactions were noted for the combination against *S. agalactiae* and *M. smegmatis*.

7.2.2.1.2 Aqueous extracts

The aqueous extracts of *Z. capense* (Table 7.2), showed the lowest MIC value with strong and noteworthy activity against *S. agalactiae* at 0.4 mg/ml. Some antimicrobial activity was seen against *C. neoformans* with a MIC value of 2.0 mg/ml.

Table 7.2 MIC and ∑FIC of *A. afra* and *Z. capense* aqueous extracts independently and in combination.

Micro-organism	**MIC (mg/ml)**			**∑FIC**	
	A. afra	***Z. capense***	***A. afra* with *Z. capense***	***A. afra* with *Z. capense* (1:1)**	**Interpretation**
K. pneumoniae NCTC 9633	12.0	13.3	12.0	1.0	Additive
M. catarrhalis ATCC 23246	8.0	16.0	8.0	0.8	Additive
E. faecalis ATCC 29212	7.0	16.0	≥16.0	ND[1]	Additive/Antagonistic[2]
C. neoformans ATCC 90112	4.0	2.0	4.0	1.5	Indifferent
S. pneumoniae ATCC 49619	8.0	≥16.0	≥16.0	ND[1]	Additive/Antagonistic[2]
S. pyogenes ATCC 8668	5.3	≥16.0	≥16.0	ND[1]	Additive/Antagonistic[2]
S. agalactiae ATCC 55618	1.7	**0.4**	1.3	2.0	Indifferent
M. smegmatis (clinical)	8.0	≥16.0	3.0	ND[1]	**Synergy**[2]

*Values in bold indicate noteworthy activity.
ND[1] No ∑FIC value could be calculated as no MIC end point for *Z. capense*/*A. afra* with *Z. capense* was obtained at the highest concentration tested.
[2] Tentative interpretation according to MIC data.
Shaded area: results previously discussed in Chapter 3 but included here for reference purposes.
Controls excluded for the sake of brevity. Please refer to Chapter 3 for further detail regarding control values.

In 2003, Motsei et al. tested the aqueous and the solvent extracts (ethanol, ethyl acetate, and hexane extracts) of *Z. capense* against *C. albicans* clinical isolates from a 5-month-old baby, an adult, and a standard strain. Results showed that the aqueous extract and the solvent extracts of the bark of *Z. capense* to be inactive against all the isolates at the highest concentration (100 mg/ml) tested.

In a study by Buwa and van Staden, (2006) that investigated the antimicrobial activity of South African traditional medicinal plants to treat venereal diseases, *Z. capense* leaf was one of the plants tested against *B. subtilis*, *E. coli*, *K. pneumoniae*, *S. aureus* as well as *Candida albicans*. The ethanol extract of *Z. capense* against *K. pneumoniae* yielded an MIC value of 3.125 mg/ml and the aqueous extract gave an MIC value of 12.5 mg/ml against the same pathogen (Buwa and van Staden, 2006). In the current study, results showed that against *K. pneumoniae*, *Z. capense* aqueous extract gave an MIC value of 13.3 mg/ml. This is consistent and comparable with the study conducted by Buwa and van Staden (2006).

The combination of *Z. capense* with *A. afra* against *S. agalactiae* yielded the highest antimicrobial activity (1.3 mg/ml). Some activity was noted against *C. neoformans* and *M. smegmatis* and very poor activity was obtained against *K. pneumoniae* and *M. catarrhalis*. No inhibition was noted against *E. faecalis*, *S. pneumoniae* and *S. pyogenes*.

∑FIC values calculated denoted indifferent interactions against *S. agalactiae* and *C. neoformans* and additivity against *K. pneumoniae* and *M. catarrhalis.* No end point MIC value was obtained for *Z. capense* against *S. pneumoniae*, *S. pyogenes* and *M. smegmatis* and in the combination against *E. faecalis*, *S. pneumoniae* and *S. pyogenes*. Thus, ∑FIC values could not be calculated. However, tentative interpretations based on the MIC values obtained were considered. (Table 7.2)

7.2.2.2 Isobologram interactions

For the combination of *A. afra* and *Z. capense* against *M. catarrhalis* (Figure 7.3), the dichloromethane: methanol extracts showed mainly additive interactions with the exception of two points at the ratio 2:8 and 1:9 (Figure 7.3, a), which proved indifferent. These points are ratios in which *Z. capense* is in the majority. For the aqueous extracts no end point MIC value was determined at the highest concentration tested thus no isobologram could be constructed. However, Figure 7.7 shows a graph depicting the MIC values of the combination

at different ratios. The results show that the MIC value increases as the ratio of *Z. capense* aqueous extract increases. The MIC value remains the same as *A. afra* alone for ratios, 9:1, 8:2, and 7:3, and then it increases from the ratio 6:4 where no MIC value was obtained at the highest concentration tested. Thus, no increase in activity is seen when the aqueous extracts of *A. afra* was combined with *Z. capense* at different ratios.

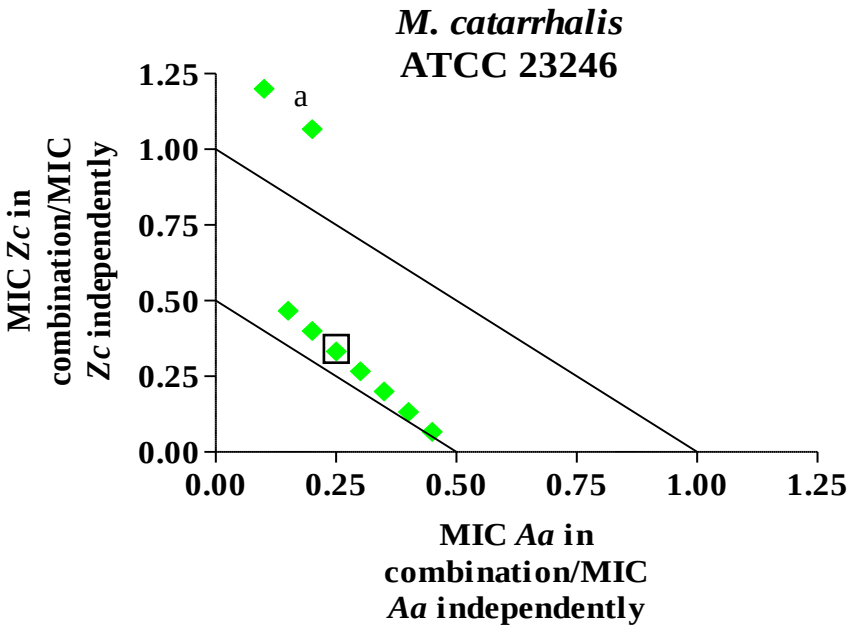


Figure 7.3 Isobologram of the combination of *A. afra* and *Z. capense* dichloromethane: methanol extracts (■) against *M. catarrhalis* (a – ratio 2:8 and 1:9); □ → 1:1 combination.

The combination against *K. pneumoniae* as shown in Figure 7.4 yielded additive interactions for all the ratios tested for the dichloromethane: methanol extracts. Interactions of the aqueous extracts are given in Figure 7.7 where the MIC values of the different ratios are given against *K. pneumoniae*. The MIC values of the ratios 9:1, 8:2, 7:3, 6:4 and 5:5 are lower than the MIC values of *A. afra* and *Z. capense* individually. Thus, a tentative interpretation of synergy is made for the interaction of these ratios of the combination. The MIC values show additive interactions at ratios 4:6, 3:7 and 2:8. It is evident that an increasing ratio of *Z capense* leads to an increase in MIC value against *K. pneumoniae*.

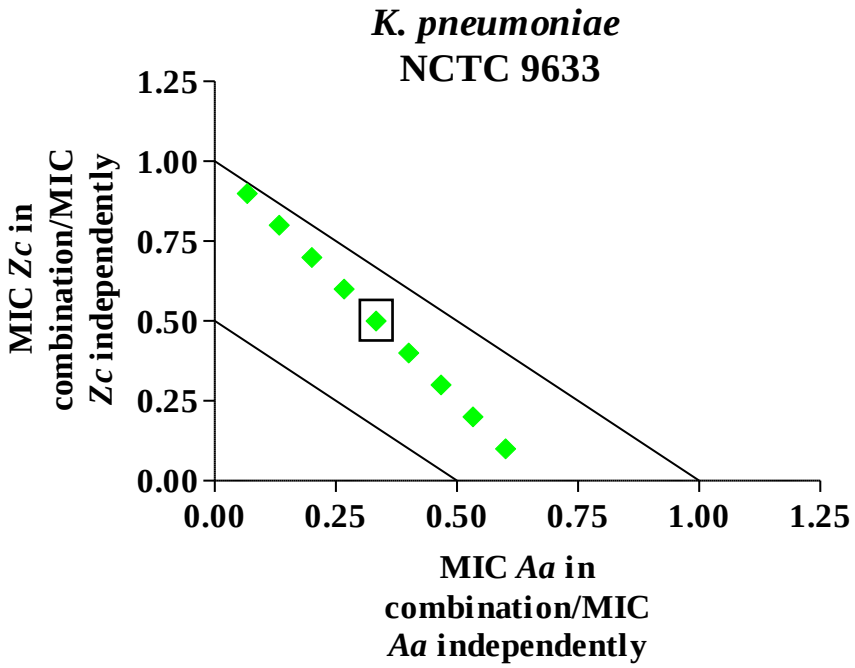


Figure 7.4 Isobologram of the combination of *A. afra* and *Z. capense* dichloromethane: methanol extracts (■) against *K. pneumoniae*; □→ 1:1 combination.

Isobologram interactions of the combination of *A. afra* and *Z. capense* against *E. faecalis* (Figure 7.5) showed mainly additive interactions for the dichloromethane: methanol extracts. One ratio was synergistic (Figure 7.5, b), i.e. 6:4, where *A. afra* was in the majority in the combination.

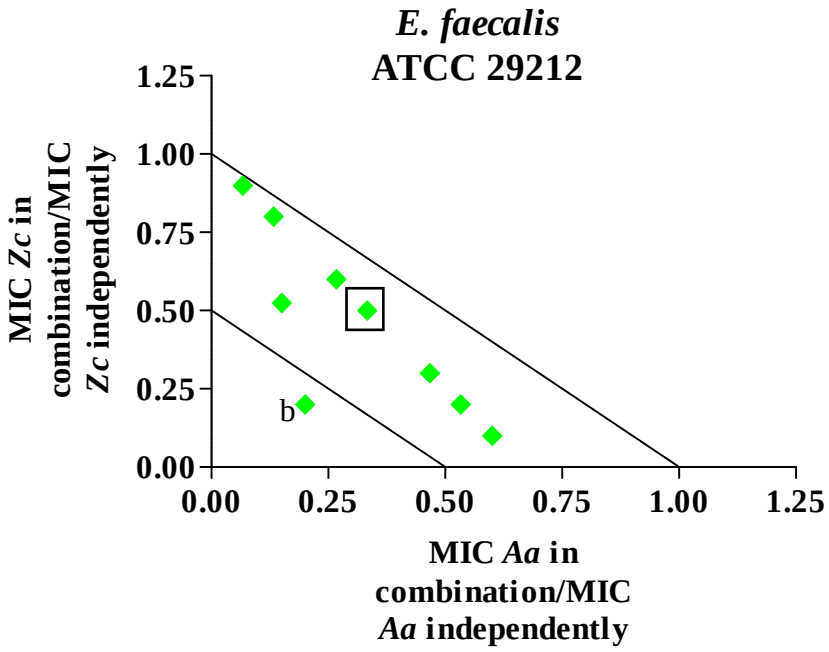


Figure 7.5 Isobologram of the combination of *A. afra* and *Z. capense* dichloromethane: methanol extracts (■) against *E. faecalis* (b – ratio 6:4); □→ 1:1 combination.

The graph depicted in Figure 7.7 shows the MIC values of the combination at different ratios, against *E. faecalis*, increasing as the ratio of *Z. capense* increases. Additivity of the combination is seen at ratios 9:1, 8:2 and 7:3, when *A. afra* is the dominant extract present. From the 1:1 ratio, the MIC value is not determinable at the highest concentration tested.

The dichloromethane: methanol extracts of the combination of *A. afra* with *Z. capense* against *C. neoformans* (Figure 7.6) showed additivity for most of the ratios tested. Synergistic activity was obtained for one ratio i.e. 7:3 (Figure 7.6, c), where *A. afra* was dominant. The aqueous extracts showed interactions including additivity and indifference. Two points, at ratios 4:6 and 1:9, where *Z. capense* was dominant, was additive. The remaining ratios tested gave indifferent pharmacological profiles.

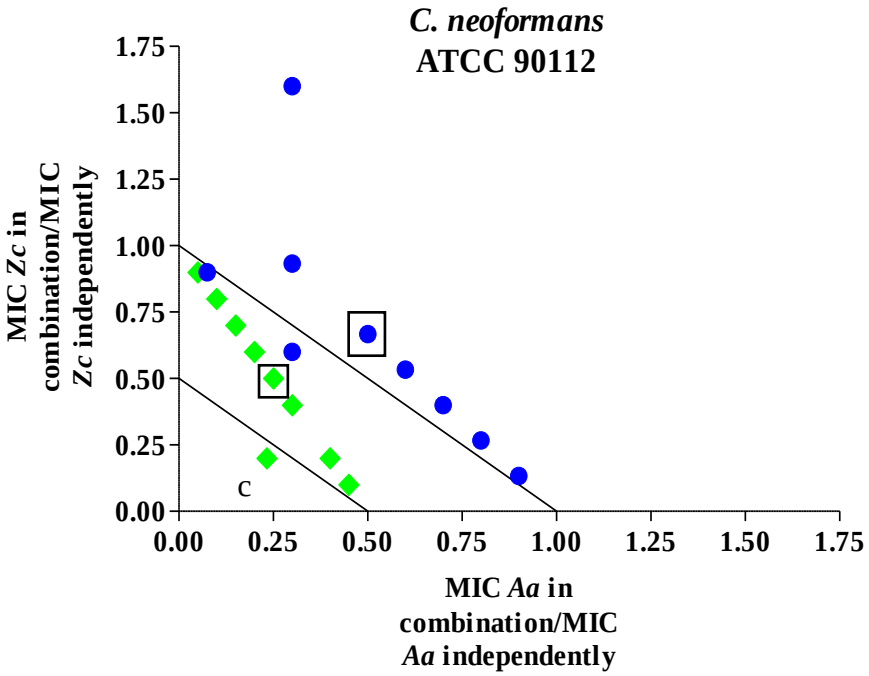


Figure 7.6 Isobologram of the combination of *A. afra* and *Z. capense* dichloromethane: methanol extracts (■) and aqueous extracts (●) against *C. neoformans* (c – ratio 7:3); □→ 1:1 combination.

Against *S. pneumoniae*, *S. pyogenes* and *M. smegmatis* the aqueous extract of *Z. capense* was not active (Table 7.2). For the aqueous extracts of the combination of *A. afra* with *Z. capense*, MIC values (Table 7.2) were not determined at the highest concentration tested against *E. faecalis*, *S. pneumoniae* and *S. pyogenes*. Tentative interpretations were carried out to determine the ∑FIC interpretations of the interactions. For the isobologram construction, the MIC values of *Z. capense* could not be calculated against *M. catarrhalis*, *K. pneumoniae* and

E. faecalis. Comparisons of the interpretations are made against the pathogens *M. catarrhalis*, *K. pneumoniae* and *E. faecalis*.

Against *M. catarrhalis*, the ∑FIC calculated (Table 7.2) was interpreted as additive and the tentative interpretation of the combination in ratios (Figure 7.7) was interpreted as being additive or indifferent. Against *K. pneumoniae*, ∑FIC interpretation was additive and the tentative interpretation of the ratios (Figure 7.7) was synergistic or additive. The ∑FIC value and the interpretation of the ratios (Figure 7.7) against *E. faecalis* could not be determined, thus tentative interpretations were carried out and both were consistent and interpreted as either additive or antagonistic.

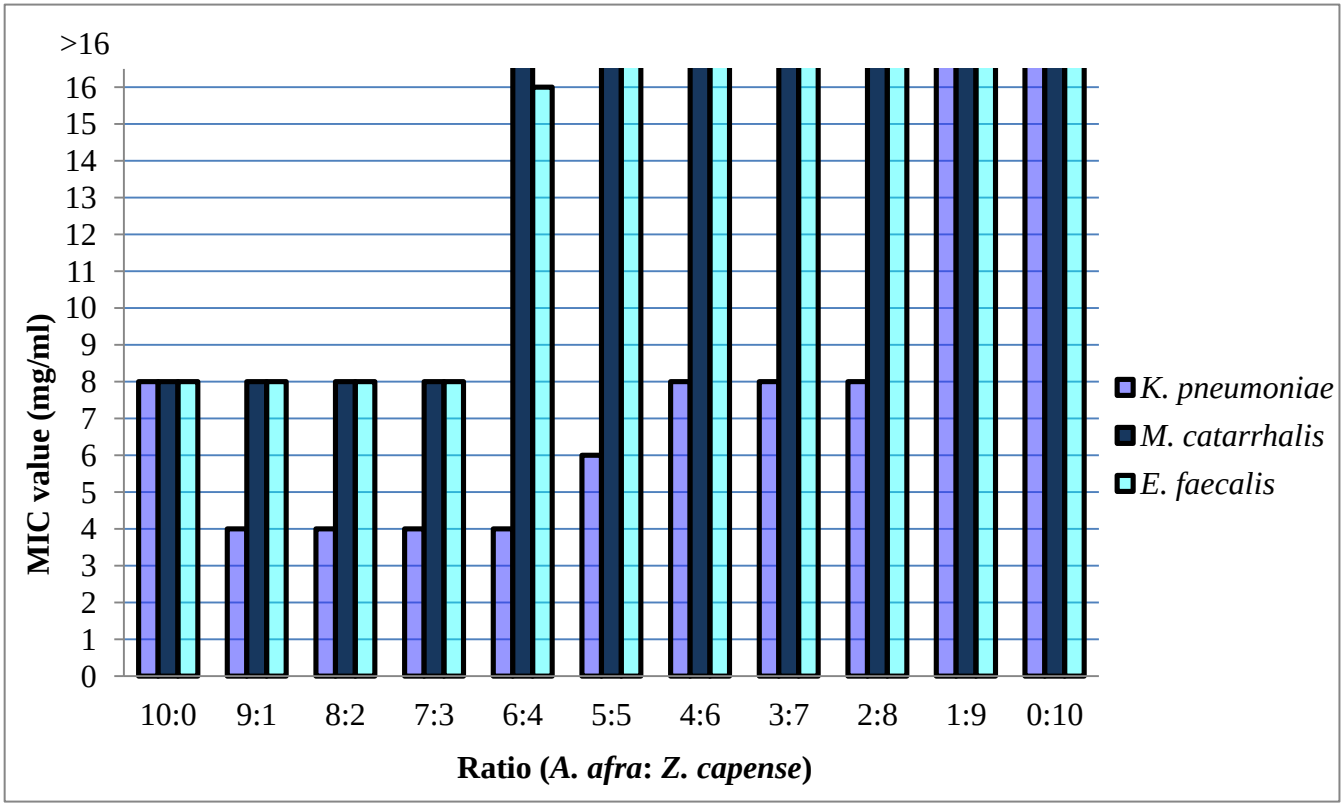


Figure 7.7 The MIC values of the different ratios of the combination of *A. afra* with *Z. capense* aqueous extracts against *K. pneumoniae*, *M. catarrhalis* and *E. faecalis.*

No other comparable studies are available to compare interactions noted between *A. afra* with *Z. capense*. The aqueous extracts of *A. afra* and *Z. capense* would represent an infusion or decoction. *Z. capense* proved inactive at the concentrations tested. However, other ways to determine if the combination is synergistic for the above-mentioned diseases or illness are available e.g. time-kill assays. Nevertheless, the isobologram results obtained give an indication that the dichloromethane: methanol extracts of *Z. capense* in combination with *A.*

afra, give synergistic and additive results depending on the ratios in which they are combined.

7.3 Conclusions

- TLC of *Z. capense* dichloromethane: methanol extracts revealed many different compounds present. Thus in the combination of *A. afra* and *Z. capense* the number of compounds present increased.
- The best antimicrobial activities noted for *Z. capense* dichloromethane: methanol extracts individually was against *C. neoformans* (MIC value = 0.4 mg/ml) and *M. smegmatis* (MIC value = 0.8 mg/ml).
- Noteworthy synergistic activity was noted in the combination of *A. afra* with *Z. capense* dichloromethane: methanol extract against *S. pneumoniae* (MIC value of 0.3 mg/ml and ∑FIC value of 0.4).
- The highest antimicrobial activity (MIC value of 0.4 mg/ml) obtained for the aqueous extracts of *Z. capense* was against *S. agalactiae.*
- The combination of *A. afra* with *Z. capense* aqueous extracts also yielded the best MIC value against *S. agalactiae* (MIC value = 1.3 mg/ml).
- Isobologram interactions revealed mainly additivity for the dichloromethane: methanol extracts against the four pathogens tested. Synergy was noted in the ratio 6:4 against *K. pneumoniae* and 7:3 against *C. neoformans*.

Chapter 8
Artemisia afra in triple plant combinations

8.1 Introduction

Western (conventional) medicine has increasingly begun using three or more agents as viable antimicrobial treatment options (Lange, 1995). This has been achieved by combining drugs like anti-retrovirals (Stavudine®-Lamivudine®-Efavirenz®), analgesics (Synap Forte®, Lentogesic®), as well as chemotherapeutic drugs (Irinotecan-fluorouracil-folinic acid) (Rossiter et al., 2010; Turner et al., 2010). A study by Ocio et al. (2010), has confirmed the efficacy of a triple combination in contrast to a double combination for the treatment of multiple myeloma. Examples of studies incorporating triple combinations include those discussed by Rochon-Edouard et al. (2000) which investigated the efficacy of β-lactams, vancomycin and netilmicin against methicillan-resistant *S. aureus* strains using time-kill studies. Results showed that the combination could, at sub-inhibitory concentrations be bactericidal after 24 hrs. These are at higher rates than a double combination of vancomycin-netilmicin. Another study of note is by O'Shaughnessy et al. (2006), where the antifungal interaction of amphotericin B, caspofungin and voriconazole was tested in combination at different drug concentrations, against *Aspergillus* species. Antifungal interactions, namely synergy, was found at low concentrations of amphotericin B (<0.2 mg/l) and voriconazole (<0.5 mg/l). Antagonism was found when the concentrations of amphotericin B and voriconazole was increased i.e. between 0.3-0.5 mg/l and >0.25 mg/l respectively. Another study pertaining to triple combinations and their uses for the treatment of *Plasmodium falciparum* infections, demonstrated no decrease in activity when folinic acid was added to a double combination (Yeo and Rieckmann, 1997). The third compound could possibly alleviate toxic effects of the other two compounds and thus prevent adverse effects normally seen in the double combination.

In order to fully explore the use of combinations in traditional medicine, it is important to recognise that plants are not only used in a two-plant combination. There are instances where three or more plants are used. Double combinations have been discussed in detail in Chapters 3-7. Typically, one would assume that the addition of a third substance to a combination would increase or improve the efficacy of a combination. However, the combinations of substances in triple or more cannot be assumed to result in synergistic or even additive

interaction. The combinations must be verified and the interaction must be established (O'Shaughnessy et al., 2006). Natural drugs or compounds are known to exhibit complex pharmacological action (Biavatti, 2009), therefore, the outcome of combining more than one or more plants is difficult to predict. Thus, it is important to test the activity in combination and thereafter have a basis upon which we may be able to scientifically validate the combinations in which they are prescribed.

Triple combinations of ethnobotanical origin are used substantially all over the world for the treatment of various conditions. Figure 8.1 shows the results obtained from an internet search on the use of triple plant combinations in general and then on triple combinations of plants in South Africa. The findings show that there is little scientific evidence on the validation of the use of triple combinations, particularly for Southern African plants. Only one validation based on southern African plants could be found, Sibandze et al., (2010), combined three Swazi traditional medicinal plants i.e. *Breonadia salicina, Syzgium cordatum* with *Ozoroa sphaerocarpa* to treat diarrhoea. Results of this study showed predominantly synergism when the three plants were combined and thus support the traditional use of the combination in Swazi traditional medicine. Other triple plant combination studies elsewhere in the world include the investigation of corni fructus, cinnamon and Chinese chive to determine the antimicrobial efficacy of the three plant extracts in combination against common food borne pathogens (Hsieh et al., 2001). Results showed that the combination 8:1:1 of the extracts produced an entire spectrum of antimicrobial activity as well as being very stable to heat and pH changes.

In South African traditional medicine, combinations of plants, either in triple or more are used frequently by traditional healers (Watt and Breyer-Brandewijk, 1962; Hutchings et al., 1996; Felhaber, 1997). Some such examples are seen in Table 1.3 and include the boiling and drinking of the combination of the powdered leaves of *Eucalyptus globulus* with powdered root of *Corbichonia decumbens* and powdered rhizome of *A. calamus* to relieve a stuffy or blocked nose. *Phoenix reclinata, Euclea natalensis, Capparis tomentosa* and *Maytennus heterophylla* are combined to treat pleurodynia and pleurisy wherein the steam from the decoction is blown into the wound (Bryant, 1970; Hutchings et al., 1996).

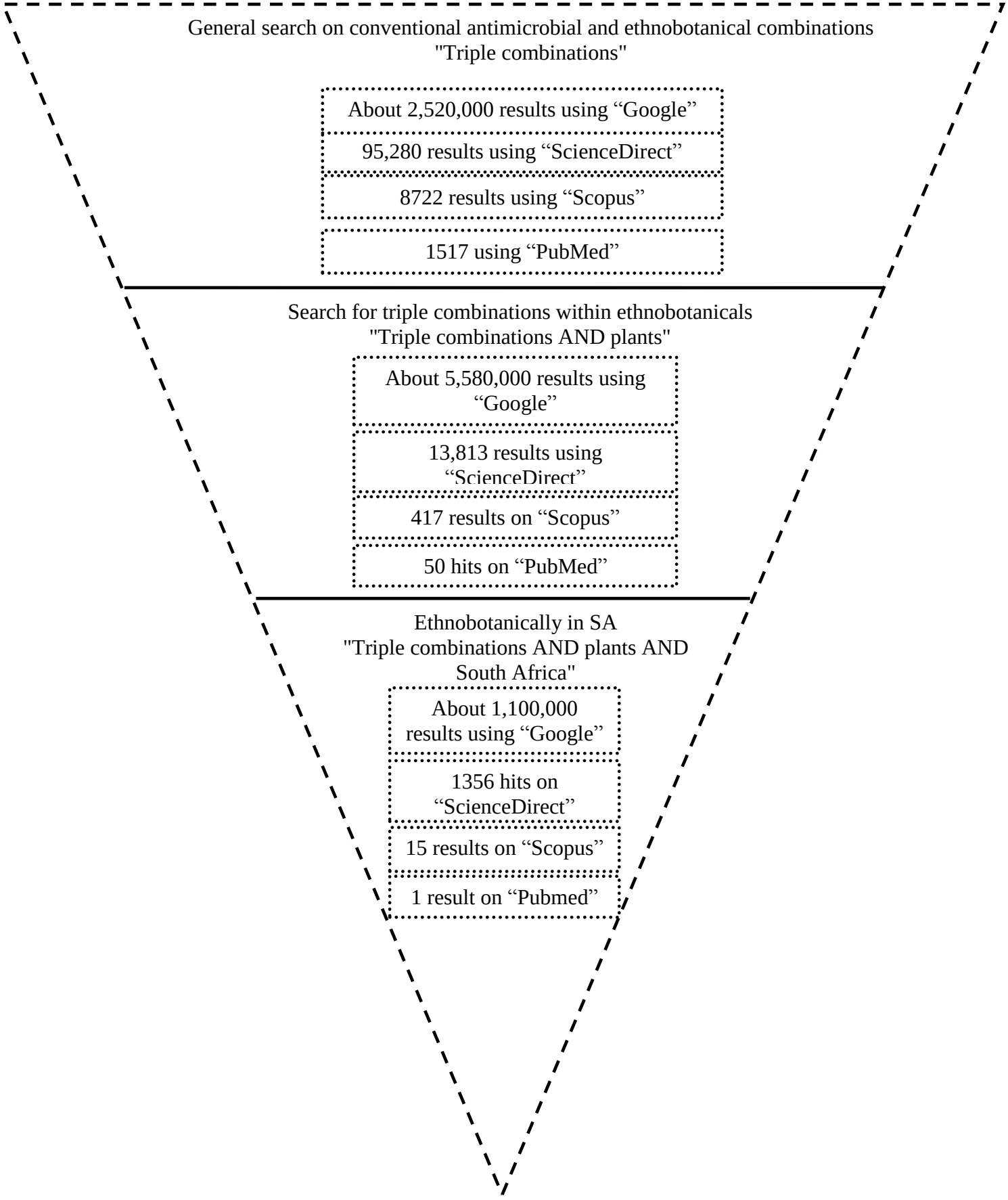


Figure 8.1 Summary of the reports available on triple combinations of medicinal plants.

A boiled infusion of the combination of *Eucalyptus globulus*, *Corbichonia decumbens* and *A. calamus* is also used for a blocked nose (Felhaber, 1997).

Combinations where *A. afra* is a component are in the treatment of asthma, wherein the root of *A. amatymbica* with the leaves of *A. afra* and the leaves of *L. leonurus* are boiled together, and taken (Felhaber, 1997). For the treatment of chronic bronchitis and emphysema, the leaves of *A. afra* are mixed with the bark of *W. salutaris* and the rhizome of *Acoras calamus*, which are then boiled and drank (Felhaber, 1997). The leaves of *A. afra*, *O. asteriscoides* with *E. globulus* were reported to be used extensively by the Europeans in South Africa for the treatment of influenza. An infusion of the leaves of *A. afra*, *Leonotis microphylla* and *E. globulus* is used by the Sotho and European people in South Africa for the relief of digestive disturbances accompanied by fever as well as for chest infections. The decoction of the combination of *A. afra*, *Allium sativum* with *Zanthoxylum capense* is used as a febrifuge (Watt and Breyer-Brandewijk, 1962).

From the literature searched, none of the afore-mentioned combinations have been scientifically validated. Moreover, evidence for synergy or antagonism between three or more plant combinations is sparse. It is important that these combinations are properly validated in order to reduce complications and adverse effects that might result if the plants used together are incompatible. Four of the triple combinations mentioned in Table 1.3 that included *A. afra* in the treatment of respiratory diseases have been selected for this study. The selection was based on the availability of the plant species, keeping in mind sustainable harvesting practices. The triple combinations evaluated are *A. afra*, *O. asteriscoides* with *E. globulus*; *A. afra*, *L. randii* with *E. globulus* and the combination of *A. afra*, *Allium sativum* with *Z. capense.*

8.2 Results and discussion

8.2.1 *A. afra, O. asteriscoides* and *E. globulus* in combination

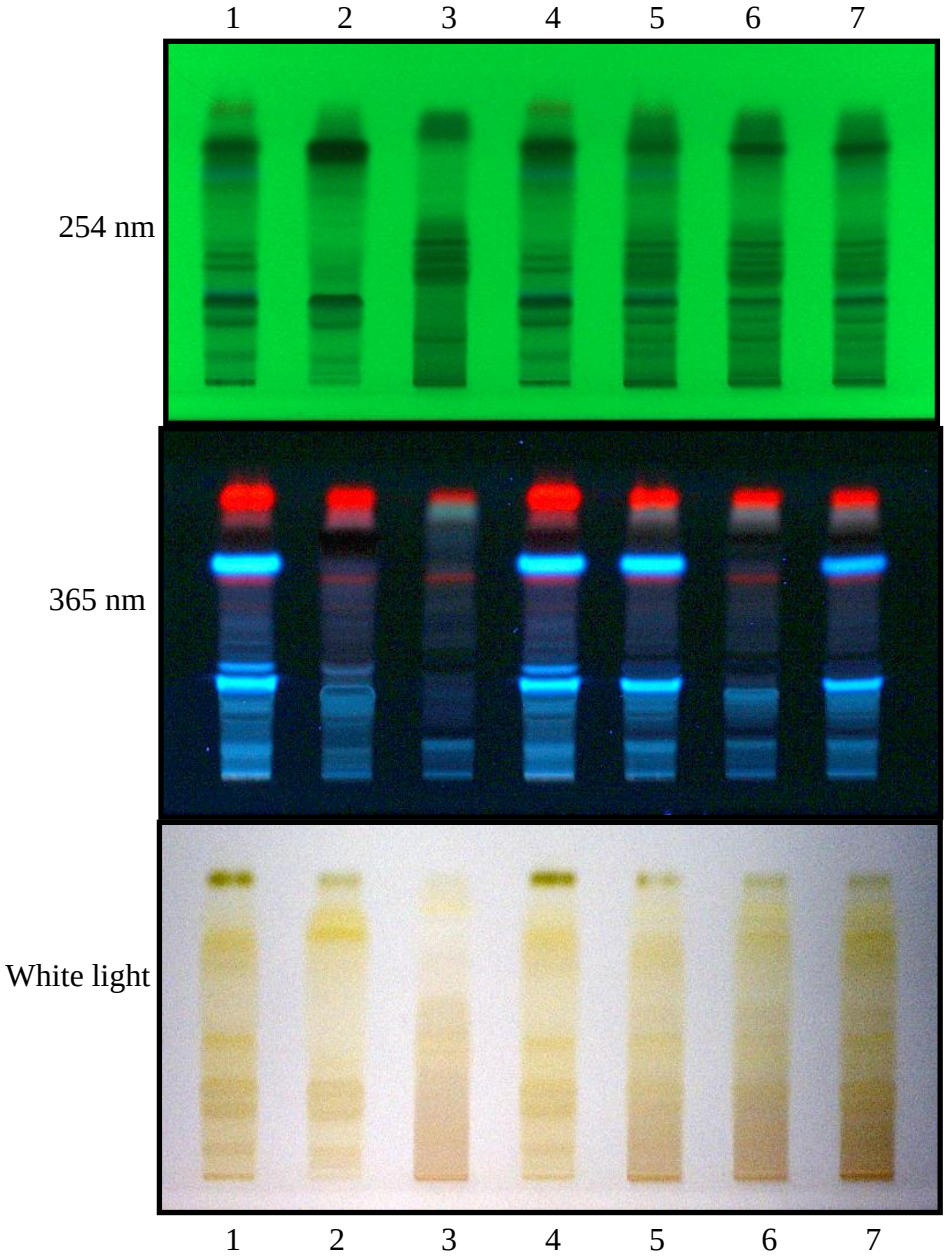
The botanical description, locality, medicinal uses and phytochemistry of *A. afra* is discussed in Chapter 1 and similarly for *E. globulus* and *O. asteriscoides* are discussed in Appendix A4 and A7, respectively. The dichloromethane: methanol extracts of *A. afra*, *O. asteriscoides* and *E. globulus* were evaluated using thin layer chromatography. MIC determination and FIC

interpretation was carried out on the essential oils, dichloromethane: methanol and the aqueous extracts according to the methods described in Chapter 2.

8.2.1.1 Chromatographic techniques

8.2.1.1.1 Thin layer chromatography

The compounds present in the plant extracts at 256 nm, individually and in combination fluoresce somewhat blackish under this wavelength due to the presence of conjugated double bonds (Figure 8.2).



1	*A. afra*
2	*O. asteriscoides*
3	*E. globulus*
4	*A. afra* with *O. asteriscoides*
5	*A. afra* with *E. globulus*
6	*O. asteriscoides* with *E. globulus*
7	*A. afra*, *O. asteriscoides* with *E. globulus*

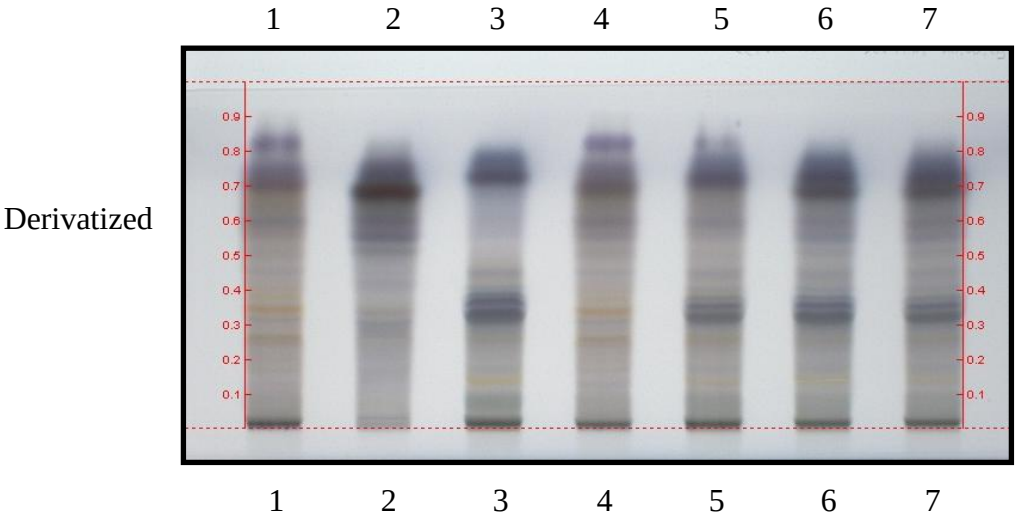


Figure 8.2 TLC plates of the dichloromethane: methanol extracts of *A. afra*, *O. asteriscoides* and *E. globulus* alone and in combination at 254 nm, 365 nm, white light and visualized with vanillin-sulphuric acid reagent.

At 365 nm (Figure 8.2), some fluorescent compounds, bright blue and red compounds, are visible. For the TLC plate viewed under white light, only the coloured compounds are detected i.e. a green compound at R_f = 0.9 in *A. afra*. When the TLC plate was derivatized with vanillin-sulphuric acid reagent, organic compounds in the test sample are coloured yellow, brown, or black (Houghton and Raman, 1998). The combination of the dichloromethane: methanol extracts of *A. afra*, *O. asteriscoides* and *E. globulus* independently and in combination thus yield compounds that are visible under UV light at 254 nm and 365 nm.

8.2.1.2 Antimicrobial analysis

8.2.1.2.1 MIC assays and FIC determination

MIC values of *A. afra*, *O. asteriscoides* and *E. globulus* individually and in combination are included in Table 8.1, 8.2 and 8.3 for the sake of completeness, (shaded areas) but have been discussed in Chapters 3, 4 and 6. The combination of *O. asteriscoides* with *E. globulus* has been studied here to give a detailed overview of each 1:1 plant combination within the triple combination. MIC values for the controls i.e. ciprofloxacin and amphotericin B are shown in Table 3.3.

8.2.1.2.1.1 Essential oils

Table 8.1 shows the MIC values of *O. asteriscoides* in combination with *E. globulus* essential oils against eight pathogens. The highest antimicrobial activity with noteworthy inhibition

was obtained for the combination of *O. asteriscoides* with *E. globulus* against *E. faecalis*, (0.5 mg/ml). Other noteworthy activity was noted for the combination against *C. neoformans* (MIC value = 0.75 mg/ml), *S. pneumoniae* (MIC value = 1.0 mg/ml), *S. pyogenes* (MIC value = 2.0 mg/ml) and *M. smegmatis* (MIC value = 1.5 mg/ml). Some antimicrobial activity was obtained in the combination against *S. agalactiae* and very low activity against *K. pneumoniae* and *M. catarrhalis*.

∑FIC values calculated for the combination of *O. asteriscoides* with *E. globulus* showed additive interactions against *K. pneumoniae, M. catarrhalis, C. neoformans S. pneumoniae, S. agalactiae* and *M. smegmatis*. The most significant synergistic activity in this triple combination study was noted against *E. faecalis* with a ∑FIC value = 0.1.

In the combination of the three essential oils i.e. *A. afra, O. asteriscoides* and *E. globulus*, noteworthy activity was obtained against *S. pneumoniae, S. pyogenes* and *M. smegmatis* (MIC value = 2.0 mg/ml). Some activity was noted against *S. agalactiae* and very poor activity was seen against *K. pneumoniae, M. catarrhalis, E. faecalis* and *C. neoformans*. ∑FIC's that were then calculated and demonstrated antagonistic activity against *C. neoformans* (∑FIC value = 6.5). Additive interactions were noted against *K. pneumoniae, M. catarrhalis, E. faecalis* and *S. agalactiae*. Against S. *pneumoniae, S. pyogenes* and *M. smegmatis* ∑FIC values showed indifferent/non-interactive interactions (∑FIC values 1.1-1.3) (Table 8.1).

In the combination of *A. afra* with *O. asteriscoides* essential oils, six of the pathogens showed additive interactions and two pathogens showed indifference. The combination of *A. afra* with *E. globulus*, all eight pathogens tested showed additive pharmacological interactions. When *O. asteriscoides* was combined with *E. globulus*, six pathogens were additive, one was indifferent and the interaction against *E. faecalis* was synergistic. When *A. afra, O. asteriscoides* and *E. globulus* essential oils were combined in a 1:1:1 ratio, four pathogens showed additive interactions, three were indifferent and one pathogen showed antagonism (*C. neoformans*).

Table 8.1 MIC and ∑FIC values of the essential oils of *A. afra*, *O. asteriscoides* and *E. globulus* alone and in combination.

Micro-organism	MIC (mg/ml)/ ∑FIC value indicated in brackets where applicable							∑FIC	
	A. afra	***O. asteriscoides***	***A. afra* with *O. asteriscoides***	***E. globulus***	***A. afra* with *E. globulus***	***O. asteriscoides* with *E. globulus***	***A. afra, O. asteriscoides* with *E. globulus***	***A. afra, O. asteriscoides* with *E. globulus***	**Interpretation**
K. pneumoniae NCTC 9633	8.0	8.0	8.0 (1.0)	8.0	8.0 (1.0)	8.0 (1.0)	8.0	1.0	Additive
M. catarrhalis ATCC 23246	8.7	8.0	8.0 (1.0)	8.0	8.0 (1.0)	8.0 (1.0)	8.0	1.0	Additive
E. faecalis ATCC 29212	8.7	8.0	8.0 (1.0)	8.0	8.0 (1.0)	**0.5** (**0.1**)	8.0	1.0	Additive
C. neoformans ATCC 90112	3.8	2.0	**2.0** (0.8)	**0.6**	**0.8** (0.8)	**0.8** (0.8)	8.0	6.5	Antagonism
S. pneumoniae ATCC 49619	3.3	**1.0**	**1.0** (0.7)	2.7	3.0 (1.0)	**1.0** (0.7)	**2.0**	1.1	Indifferent
S. pyogenes ATCC 8668	**1.7**	**1.7**	**1.5** (0.9)	**2.0**	**1.5** (0.8)	**2.0** (1.1)	**2.0**	1.1	Indifferent
S. agalactiae ATCC 55618	4.8	4.7	7.0 (1.5)	5.3	4.0 (0.8)	4.0 (0.8)	4.0	0.8	Additive
M. smegmatis (clinical)	**1.5**	**1.3**	**1.5** (1.1)	**2.0**	**1.3** (0.7)	**1.5** (1.0)	**2.0**	1.3	Indifferent

*Values in bold indicate noteworthy activity.
Shaded area: results previously discussed in Chapter 3, 4 and 5 but included here for reference purposes.
Controls excluded for the sake of brevity. Please refer to Chapter 3 for further detail regarding control values.

The triple combination did not show any marked increase in activity or synergy when compared to the double combinations. In fact, against *C. neoformans*, it is imperative that the three oils are not combined as detrimental effects could result due to the antagonism they present. Inhalation is not used when administering this combination thus it was not surprising that the combination did not show any marked improvement in interaction. One combination that stands out in the combinations involving the essential oils is *O. asteriscoides* with *E. globulus* against *E. faecalis* where the most significant synergistic interaction was observed for the entire study.

8.2.1.2.1.2 Dichloromethane: methanol extracts

For the dichloromethane: methanol extracts, MIC values of the combination of *O. asteriscoides* with *E. globulus* are shown in Table 8.2. Noteworthy inhibitory activity was seen against *C. neoformans* (MIC value = 0.3 mg/ml), *S. pneumoniae* (MIC value = 0.5 mg/ml) and *S. agalactiae* (MIC value = 0.5 mg/ml). Moderate inhibition was noted against *S. pyogenes* with a MIC value of 1.0 mg/ml.

Some antimicrobial activity was seen against *K. pneumoniae*, *M. catarrhalis*, *E. faecalis* and *M. smegmatis*. The ∑FIC values calculated for the combination of *O. asteriscoides* with *E. globulus* gave additive interactions against *K. pneumoniae* and *C. neoformans* with ∑FIC values of 0.7 and 0.8 respectively. Synergy was obtained against *S. agalactiae* where the ∑FIC calculated was 0.4. Antagonism was observed for studies against *M. catarrhalis* and *E. faecalis.*

For the triple combination of the plants extracts, MIC values (0.5 mg/ml) demonstrated noteworthy strong inhibition against *C. neoformans* and *S. agalactiae*. Noteworthy moderate inhibition was observed for *S. pneumoniae* (MIC value = 0.6 mg/ml). Moderate inhibitory antimicrobial activity was obtained against *S. pyogenes* with a MIC value of 1.0 mg/ml.

Some activity was noted against *K. pneumoniae*, *M. catarrhalis*, *E. faecalis* and *M. smegmatis*. ∑FIC values of the triple combination yielded synergistic interactions against two pathogens i.e. against *K. pneumoniae* (∑FIC value = 0.5) and *S. agalactiae* (∑FIC value = 0.4). Two pathogens revealed complete antagonism when the ∑FIC values were calculated. These were *M. catarrhalis* and *E. faecalis*. One combination was additive against *C. neoformans* (∑FIC value = 0.9).

Table 8.2 MIC and ∑FIC values of the dichloromethane: methanol extracts of *A. afra*, *O. asteriscoides* and *E. globulus* alone and in combination.

Micro-organism	**MIC (mg/ml)/∑FIC value indicated in brackets where applicable**							**∑FIC**	
	A. afra	***O. asteriscoides***	***A. afra* with *O. asteriscoides***	***E. globulus***	***A. afra* with *E. globulus***	***O. asteriscoides* with *E. globulus***	***A. afra, O. asteriscoides* with *E. globulus***	***A. afra, O. asteriscoides* with *E. globulus***	**Interpretation**
K. pneumoniae NCTC 9633	6.3	4.0	6.0 (1.2)	3.0	2.0 (0.5)	2.0 (0.7)	2.0	**0.5**	**Synergism**
M. catarrhalis ATCC 23246	4.7	4.0	4.0 (0.9)	0.01	1.1 (5.8)	2.2 (77.3)	4.0	57.6	Antagonism
E. faecalis ATCC 29212	6.3	2.0	4.0 (1.3)	0.02	2.1 (7.6)	1.1 (34.0)	2.0	42.9	Antagonism
C. neoformans ATCC 90112	1.2	0.3	0.3 (0.7)	0.5	0.8 (1.1)	**0.3 (0.8)**	**0.5**	0.9	Additive
S. pneumoniae ATCC 49619	0.5	0.4	0.1 (0.3)	0.19	0.3 (0.9)	**0.5 (1.9)**	**0.6**	1.9	Indifferent
S. pyogenes ATCC 8668	1.1	0.4	0.1 (0.2)	0.6	0.3 (0.3)	1.0 (2.1)	1.0	1.7	Indifferent
S. agalactiae ATCC 55618	3.0	1.3	1.0 (0.6)	1.0	1.0 (0.7)	**0.5 (0.4)**	**0.5**	**0.4**	**Synergism**
M. smegmatis (clinical)	1.7	1.4	0.5 (0.3)	1.5	0.5 (0.3)	2.0 (1.4)	2.0	1.3	Indifference

*Values in bold indicate noteworthy activity.
Shaded area: results previously discussed in Chapter 3, 4 and 5 but included here for reference purposes.
Controls excluded for the sake of brevity. Please refer to Chapter 3 for further detail regarding control values.

The combination of the dichloromethane: methanol extracts of *A. afra* with *O. asteriscoides* showed varied interactions .The combination of *A. afra* with *E. globulus* demonstrated three synergistic interactions (against *K. pneumoniae*, *S. pyogenes* and *M. smegmatis*) and two antagonistic (*M. catarrhalis* and *E. faecalis*) interactions.. For the combination of *O. asteriscoides* with *E. globulus*, only one interaction was evident and this was against *S. agalactiae*. The triple combination of *A. afra* with *O. asteriscoides* and *E. globulus* demonstrated varied interactions with two synergistic interactions against *K. pneumoniae* and *S. agalactiae*. The triple combination is thus better than the double combinations when treating *K. pneumoniae* and *S. agalactiae* relating infections. However, care should be taken when treating *M. catarrhalis* and *E. faecalis* infections due to the antagonism encountered with these two pathogens. Thus, the causative micro-organism should be identified before treatment is administered to avoid any unwanted side effects.

8.2.1.2.1.3 Aqueous extracts

The MIC values of the aqueous extracts (Table 8.3) of the combination of *O. asteriscoides* with *E. globulus* demonstrated moderate inhibition against *S. agalactiae* (MIC value = 1.0 mg/ml). Some antimicrobial activity was obtained against *M. catarrhalis*, *E. faecalis*, *C. neoformans*, *S. pneumoniae*, *S. pyogenes* and *M. smegmatis*. The aqueous extracts showed additive ∑FIC values of 0.8, 1.0, 0.7 and 1.0 against *M. catarrhalis*, *E. faecalis*, *S. agalactiae* and *M. smegmatis* respectively. Against *K. pneumoniae*, *C. neoformans*, *S. pneumoniae* and *S. pyogenes* indifferent interactions were noted.

The MIC values of the triple combination ranged from 2.0 - 6.0 mg/ml with *K. pneumoniae* having the least sensitivity to the combination (MIC value of 6.0 mg/ml). In contrast, the triple combination was most active against *S. pyogenes* and *S. agalactiae* with MIC values of 2.0 mg/ml. The ∑FIC values of the triple combination against *K. pneumoniae*, *M. catarrhalis*, *E. faecalis*, *S. pyogenes* and *M. smegmatis* all demonstrate additive interactions i.e. ∑FIC values of 0.8, 0.7, 0.9, 1.0 and 0.8 respectively. ∑FIC values calculated against *C. neoformans*, *S. pneumoniae* and *S. agalactiae* showed indifferent interactions when the aqueous extracts of all three plants were combined.

Table 8.3 MIC and ∑FIC values of the aqueous extracts of *A. afra*, *O. asteriscoides* and *E. globulus* alone and in combination.

Micro-organism	MIC (mg/ml)/∑FIC value indicated in brackets where applicable							∑FIC	
	A. afra	***O. asteriscoides***	***A. afra* with *O. asteriscoides***	***E. globulus***	***A. afra* with *E. globulus***	***O. asteriscoides* with *E. globulus***	***A. afra, O. asteriscoides* with *E. globulus***	***A. afra, O. asteriscoides* with *E. globulus***	**Interpretation**
K. pneumoniae NCTC 9633	12.0	8.0	8.0 (0.8)	6.0	8.0 (1.0)	8.0 (1.2)	6.0	0.8	Additive
M. catarrhalis ATCC 23246	8.0	8.0	8.0 (1.0)	3.5	4.0 (0.8)	4.0 (0.8)	4.0	0.7	Additive
E. faecalis ATCC 29212	7.0	9.0	8.0 (1.0)	2.5	4.0 (1.1)	4.0 (1.0)	4.0	0.9	Additive
C. neoformans ATCC 90112	4.0	0.5	0.5 (0.6)	0.8	0.4 (0.3)	2.0 (3.3)	3.0	2.3	Indifferent
S. pneumoniae ATCC 49619	8.0	1.5	2.0 (0.8)	14.0	2.0 (0.2)	4.0 (1.5)	4.0	1.2	Indifferent
S. pyogenes ATCC 8668	5.3	1.8	1.5 (0.6)	1.3	2.0 (0.9)	2.0 (1.3)	2.0	1.0	Additive
S. agalactiae ATCC 55618	1.7	1.5	2.0 (1.3)	1.5	1.0 (0.6)	1.0 (0.7)	2.0	1.3	Indifferent
M. smegmatis (clinical)	8.0	4.0	4.0 (0.8)	4.0	4.0 (0.8)	4.0 (1.0)	4.0	0.8	Additive

*Values in bold indicate noteworthy activity.
Shaded area: results previously discussed in Chapter 3, 4 and 5 but included here for reference purposes.
Controls excluded for the sake of brevity. Please refer to Chapter 3 for further detail regarding control values.

The aqueous extracts of the combination of *A. afra* with *O. asteriscoides* yielded interactions against seven pathogens to be additive and one indifferent. The combination of the extracts of *A. afra* with *E. globulus* showed varied interactions with, two noteworthy synergistic (against *C. neoformans* and *S. pneumoniae*) profiles. For the combination of *O. asteriscoides* with *E. globulus*, indifferent number of varied interactions were also noted, none of which were antagonistic. The triple combination is administered as an infusion (van Wyk and Wink, 2004). The results obtained correlate with the traditional use and the advantage of the administration of the triple combination can be seen, as none of the interactions obtained against the pathogens demonstrated any adverse effects.

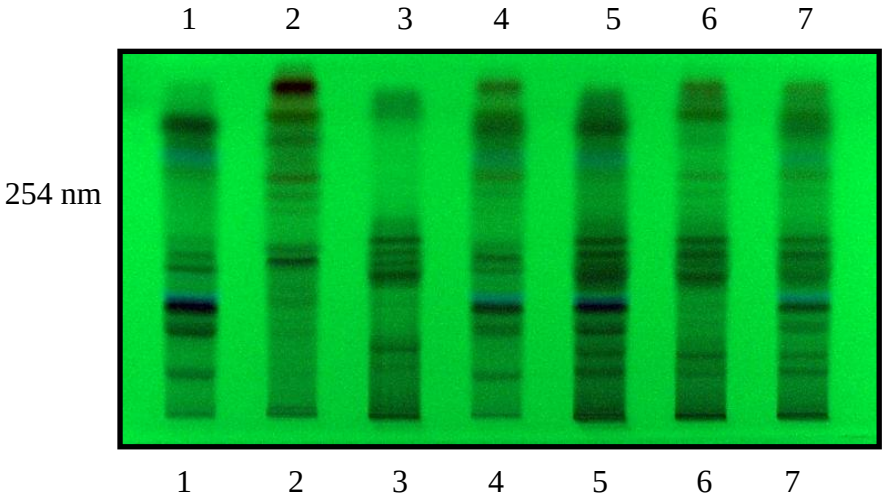
8.2.2 *A. afra, E. globulus* and *L. randii* in combination

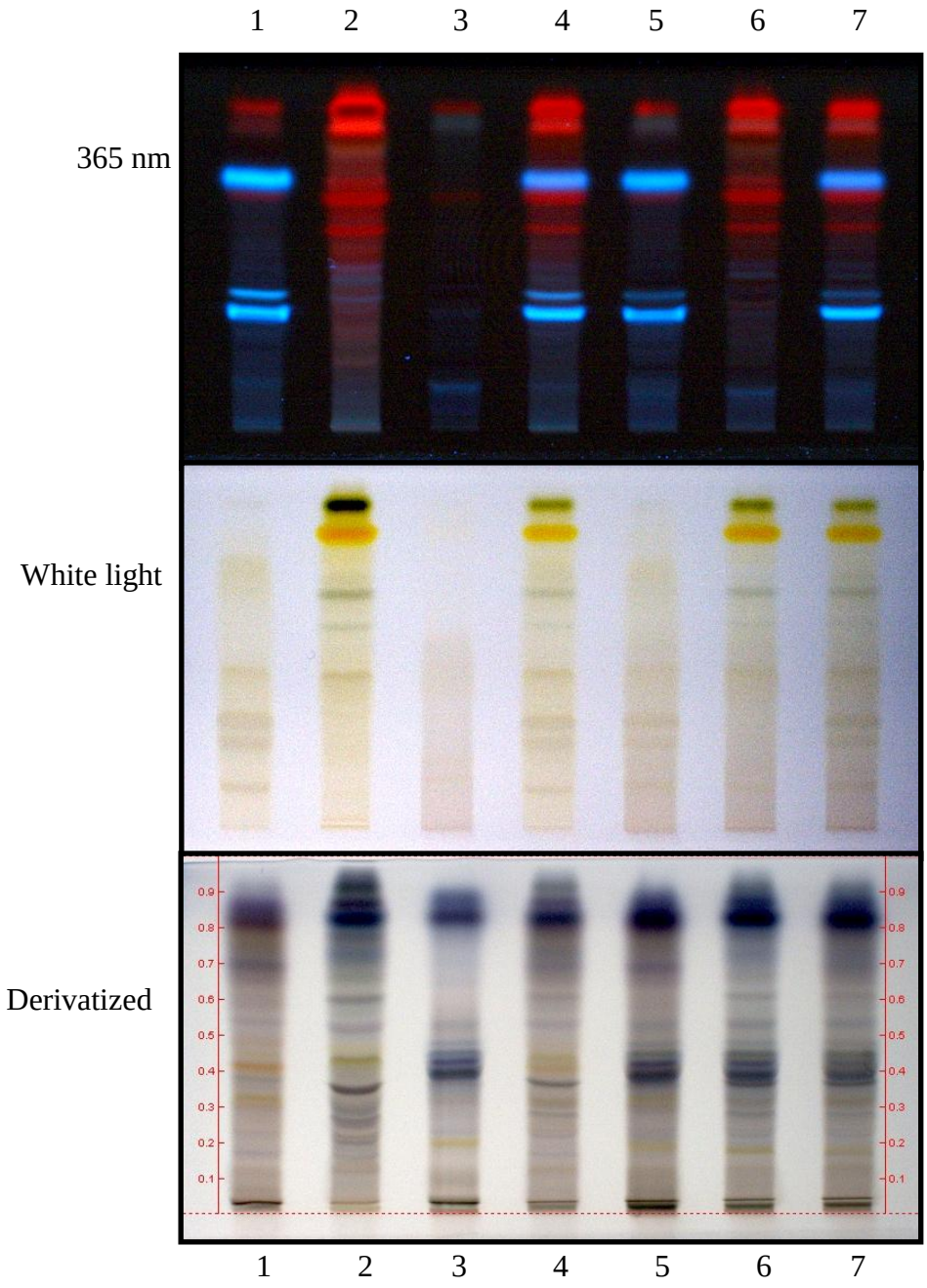
The botanical description, locality, medicinal uses and phytochemistry of *A. afra* is discussed in Chapter 3 and similarly of *E. globulus* and *L. randii* are discussed in Appendix A4 and A5. The dichloromethane: methanol extracts of *A. afra, E. globulus* with *L. randii* were evaluated using thin layer chromatography. MIC determination and ∑FIC interpretation was carried out on the dichloromethane: methanol and the aqueous extracts according to the methods described in Chapter 2. Although *L. randii* is an aromatic plant, the essential oils were not studied as no oil was obtained during the hydrodistillation process, and thus have been excluded in the triple combination study.

8.2.2.1 Chromatographic techniques

8.2.2.1.1 Thin layer chromatography

Thin layer chromatography analysis of the dichloromethane: methanol extracts of *A. afra, E. globulus*, and *L. randii* shows conjugated double bonded compounds, which are highly fluorescent at 254 nm (Figure 8.3).





1	*A. afra*
2	*L. randii*
3	*E. globulus*
4	*A. afra* with *L. randii*
5	*A. afra* with *E. globulus*
6	*L. randii* with *E. globulus*
7	*A. afra*, *L. randii* with *E. globulus*

Figure 8.3 TLC plates of the dichloromethane: methanol extracts of *A. afra*, *L. randii* and *E. globulus* alone and in combination at 254 nm, 365 nm, white light and visualized with vanillin-sulphuric acid reagent.

At 365 nm, *L. randii* displayed many compounds, which showed up red and blue in colour. Under white light (Figure 8.3), *L. randii* showed the most coloured compound of yellow. This compound was found between R_f values 0.8 and 0.9.

8.2.2.2 Antimicrobial analysis

8.2.2.2.1 MIC assays and FIC determination

8.2.2.2.1.1 Dichloromethane: methanol extracts

The MIC values of *A. afra*, *L. randii* and *E. globulus* dichloromethane: methanol extracts against the various pathogens tested are shown in Table 8.4. MIC values individually of *L. randii* showed noteworthy activity against *M. smegmatis* with a MIC value of 0.5 mg/ml. when tested against *K. pneumoniae* and *S. pyogenes*, weak inhibition was noted with mean MIC values of 2.0 mg/ml and 1.0 mg/ml respectively. Moderate inhibitory antimicrobial activity was noted against *M. catarrhalis* (MIC value = 1.3 mg/ml), *E. faecalis* (MIC value = 1.5 mg/ml) and *S. pneumoniae* (MIC value = 1.5 mg/ml).Very poor activity was seen against *C. neoformans* and *S. agalactiae* with MIC values of 8.0 mg/ml and 12.0 mg/ml.

In the combination of the dichloromethane: methanol extracts of *A. afra* and *L. randii* mean MIC values ranged from 0.3-8.0 mg/ml. *C. neoformans* and *M. smegmatis* gave the best MIC values, with noteworthy strong inhibition, of 0.3 mg/ml and 0.5 mg/ml respectively. Against *S. pyogenes*, the poorest activity of the combination was noted at 8.0 mg/ml. Moderate inhibitory activity was noted against *S. pneumoniae* with a mean MIC value of 1.0 mg/ml. Some activity was seen against *K. pneumoniae*, *M. catarrhalis*, *E. faecalis* and *S. agalactiae*. The ∑FIC values, in brackets (Table 8.4) showed synergy for *C. neoformans* with a ∑FIC value of 0.14. Additive interactions were noted against *M. catarrhalis*, *E. faecalis*, *S. agalactiae* and *M. smegmatis*.

A study was recently conducted (Eloff, 2010) testing the antimicrobial activity of the acetone extract of *L. randii* against four pathogens i.e. *S. aureus*, *P. aeruginosa*, *E. coli* and *E. faecalis*. Results showed good antimicrobial activities with MIC values below 0.1 mg/ml against *S. aureus* (0.058 mg/ml), *P. aeruginosa* (0.033 mg/ml) and *E. faecalis* (0.015 mg/ml). The MIC value against *E. coli* was 0.113 mg/ml. The study concluded that after toxicity and stability studies are carried out, *L. randii* could be promising as a commercial antibacterial agent.

The combination of *E. globulus* and *L. randii* showed noteworthy inhibitory antimicrobial activity against *K. pneumoniae* (MIC value = 0.5 mg/ml), *C. neoformans* (MIC value = 0.5 mg/ml), *S. pneumoniae* (MIC value = 0.3 mg/ml) and *M. smegmatis* (MIC value = 0.5 mg/ml).

Table 8.4 MIC and ∑FIC values of the dichloromethane: methanol extracts of *A. afra*, *L. randii*, and *E. globulus* alone and in combination.

Micro-organism	MIC (mg/ml)/ ∑FIC value indicated in brackets where applicable							∑FIC	
	A. afra	***L. randii***	***A. afra* with *L. randii***	***E. globulus***	***A. afra* with *E. globulus***	***L. randii* with *E. globulus***	***A. afra, L. randii* with *E. globulus***	***A. afra, L. randii* and *E. globulus***	**Interpretation**
K. pneumoniae NCTC 9633	6.3	2.0	4.0 (1.3)	3.0	2.0 (0.5)	**0.5 (0.23)**	**0.8**	**0.3**	**Synergy**
M. catarrhalis ATCC 23246	4.7	1.3	2.0 (1.0)	**0.01**	1.1 (5.8)	1.4 (36.55)	**0.04**	1.0	Additive
E. faecalis ATCC 29212	6.3	1.5	2.0 (0.8)	**0.02**	2.1 (7.6)	**1.0** (25.1)	**0.04**	0.8	Additive
C. neoformans ATCC 90112	1.2	8.0	**0.3 (0.1)**	**0.5**	**0.8** (1.1)	**0.5** (0.5)	**0.3**	**0.3**	**Synergy**
S. pneumoniae ATCC 49619	**0.5**	1.5	1.0 (1.3)	**0.2**	**0.3** (0.9)	**0.3** (0.9)	**0.3**	0.8	Additive
S. pyogenes ATCC 8668	1.1	1.7	8.0 (3.0)	**0.6**	**0.3 (0.3)**	1.0 (1.1)	**0.8**	0.8	Additive
S. agalactiae ATCC 55618	3.0	12.0	4.0 (0.8)	1.0	1.0 (0.7)	2.0 (1.1)	8.0	3.8	Indifference
M. smegmatis (clinical)	1.7	**0.5**	**0.5** (0.7)	1.5	**0.5 (0.3)**	**0.5** (0. 7)	**0.5**	**0.5**	**Synergy**

*Values in bold indicate noteworthy activity
Shaded area: results previously discussed in Chapter 3 and 6 but included here for reference purposes.
Controls excluded for the sake of brevity. Please refer to Chapter 3 for further detail regarding control values.

Noteworthy moderate inhibition was obtained against *E. faecalis* with a MIC value of 0.99 mg/ml. Some inhibition was seen against *M. catarrhalis*, *S. pyogenes* and *S. agalactiae*. ∑FIC values for this combination gave synergistic activity against *K. pneumoniae* (∑FIC value = 0.23) and additive interactions against *C. neoformans*, *S. pneumoniae* and *M. smegmatis*. Antagonism was noted against *M. catarrhalis* and *E. faecalis*.

The triple combination, *A. afra* with *E. globulus* and *L. randii*, yielded noteworthy activity against all pathogens tested except against *S. agalactiae*, which gave very poor activity with an MIC value of 8.0 mg/ml. The best antimicrobial activity, and very strong inhibition, of the triple combination was against *M. catarrhalis* and *E. faecalis* with mean MIC values of 0.04 mg/ml. Against *K. pneumoniae* and *S. pyogenes* moderate inhibitory activity was noted, while against *C. neoformans*, *S. pneumoniae* and *M. smegmatis*, strong inhibition was evident. ∑FIC values of the triple combination thus gave additive interactions against *M. catarrhalis*, *E. faecalis*, *S. pneumoniae* and *S. pyogenes*. Synergistic inhibition was obtained against *K. pneumoniae*, *C. neoformans* and *M. smegmatis*.

Comparisons of this study with that of *L. randii* acetone extracts (Eloff, 2010) showed some variation. This variation could be due to the difference in extract prepared or due to different plant collection sites.

For the combination of *A. afra* with *L. randii* (dichloromethane: methanol extracts) against the eight pathogens tested, four of them demonstrated additive interactions with one antagonistic (*S. pyogenes*) and one synergistic (*C. neoformans*). The interactions for the combination of *A. afra* with *E. globulus* gave three synergistic interactions against *K. pneumoniae*, *S. pyogenes* and *M. smegmatis*. Two pathogens displayed antagonism (*M. catarrhalis* and *E. faecalis*). The combination of *L. randii* with *E. globulus* gave one synergistic interaction against *K. pneumoniae*, three additive interactions and two antagonistic interactions (*M. catarrhalis* and *E. faecalis*). The combination of *A. afra* with *L. randii* and *E. globulus* displayed varied interactions, depending on the pathogen studied. The triple combination is better than the double combinations as no antagonism was noted and positive results (synergistic and additive interactions) were obtained. The traditional use of the triple combination is cited as being administered as an infusion. However, due to the results obtained, the use of a tincture might be a more viable option.

8.2.2.2.1.2 Aqueous extracts

The aqueous extracts of *L. randii* independently, as shown in Table 8.5, proved ineffective in inhibiting *E. faecalis*, *S. pneumoniae*, and *S. pyogenes* at the highest concentration tested. Some activity was noted against *K. pneumoniae*, *C. neoformans*, *S. agalactiae* and *M. smegmatis* and very poor activity was obtained against *M. catarrhalis*.

For the combination of *A. afra* with *L. randii*, no activity at the highest concentration tested was noted against *S. pneumoniae* and *S. pyogenes*. Some activity was noted against *K. pneumoniae*, *E. faecalis*, *C. neoformans*, *S. agalactiae* and *M. smegmatis*. Very poor activity was seen against *M. catarrhalis*, with a MIC value of 12.0 mg/ml. ∑FIC values calculated for the combination gave additive interactions against *K. pneumoniae*, *C. neoformans* and *M. smegmatis*. Tentative interpretations against *S. pneumoniae* and *S. pyogenes* showed additive or antagonistic interactions present.

In the combination of *E. globulus* with *L. randii* aqueous extract moderate inhibitory activity with MIC values of 1.0 mg/ml was obtained against *C. neoformans* and *S. pneumoniae*. Noteworthy strong inhibition was obtained against *M. smegmatis* (MIC value = 0.5 mg/ml). ∑FIC values calculated showed additive interactions of *E. globulus* with *L. randii* aqueous extract against *K. pneumoniae* and *C. neoformans*. A synergistic interaction against *M. smegmatis* was shown with a ∑FIC value = 0.2. The combination against *S. agalactiae* was interpreted as being antagonistic.

In the combination of the three plants together i.e. *A. afra*, *E. globulus* with *L. randii*, very poor activity with a MIC value of 16.0 mg/ml and 8.0 mg/ml was noted against *S. pneumoniae* and *M. catarrhalis* respectively. Some activity was seen against *K. pneumoniae*, *E. faecalis*, *C. neoformans*, *S. agalactiae* and *M. smegmatis*. The ∑FIC values calculated, showed an additive interaction against *K. pneumoniae*. Tentative interpretations of the interactions of the triple combination showed synergistic or additive activity against *E. faecalis* and additive or indifferent activity against *S. pneumoniae*.

Table 8.5 MIC and ∑FIC values of the aqueous extracts of *A. afra*, *L. randii*, and *E. globulus* alone and in combination.

Micro-organism	MIC (mg/ml)/ ∑FIC value indicated in brackets where applicable							∑FIC	
	A. afra	***L. randii***	***A. afra* with *L. randii***	***E. globulus***	***A. afra* with *E. globulus***	***L. randii* with *E. globulus***	***A. afra, L. randii* with *E. globulus***	***A. afra, L. randii* with *E. globulus***	**Interpretation**
K. pneumoniae NCTC 9633	12.0	6.0	6.0 (0.8)	6.0	8.0 (1.0)	6.0 (1.0)	4.0	0.7	Additive
M. catarrhalis ATCC 23246	8.0	8.0	12.0 (1.5)	3.5	4.0 (0.8)	8.0 (1.6)	8.0	1.3	Indifferent
E. faecalis ATCC 29212	7.0	≥16.0	2.0 (ND[1])	2.5	4.0 (1.1)	2.0 (ND[1])	2.0	ND[1]	Synergistic/Additive[2]
C. neoformans ATCC 90112	4.0	6.0	4.0 (0.8)	**0.8**	**0.4 (0.3)**	1.0 (0.7)	2.0	1.1	Indifferent
S. pneumoniae ATCC 49619	8.0	≥16.0	≥16.0 (ND[1])	14.0	2.0 (**0.2**)	1.0 (ND[1])	16.0	ND[1]	Additive/Indifferent[2]
S. pyogenes ATCC 8668	5.3	≥16.0	≥16.0 (ND[1])	1.3	2.0 (0.9)	6.0 (ND[1])	≥16.0	ND[1]	Antagonism/Indifferent[2]
S. agalactiae ATCC 55618	1.7	4.0	4.0 (1.7)	1.5	1.0 (0.6)	≥16.0 (ND[1])	4.0	1.6	Indifferent
M. smegmatis (clinical)	8.0	2.0	3.0 (0.9)	4.0	4.0 (0.8)	**0.5 (0.2)**	4.0	1.4	Indifferent

*Values in bold indicate noteworthy activity.
ND[1] No ∑FIC value could be calculated as no MIC end point for *L. randii*/combination was obtained at the highest concentration tested.
[2] Tentative interpretation according to MIC data.
Shaded area: results previously discussed in Chapter 3 and 6 but included here for reference purposes.
Controls excluded for the sake of brevity. Please refer to Chapter 3 for further detail regarding control values.

The combination of *A. afra* with *L. randii* aqueous extract displayed three additive interactions against the pathogens tested. Tentative interpretations showed additive or antagonistic interactions against *S. pneumoniae* and *S. pyogenes* respectively. Synergy was also noted against the pathogen *E. faecalis*. For the combination of *A. afra* with *E. globulus*, the interactions against five pathogens showed additivity and against two pathogens synergism was noted (*C. neoformans* and *S. pneumoniae*). The interactions observed for the combination of *E. globulus* with *L. randii* against one pathogen was synergistic (*M. smegmatis*). Tentatively interpretations against *E. faecalis* were synergistic or additive. Against *S. pneumoniae* synergy was noted and against *S. pyogenes* additivity or indifference was observed. The interaction against *S. agalactiae* tentatively displayed antagonism. The triple combination, *A. afra* with *L. randii* and *E. globulus* displayed an interaction against one pathogen which was additive. Tentatively, synergy or additivity was noted against *E. faecalis.*

Traditionally infusions of the triple combination of *A. afra* with *L. randii* and *E. globulus* are used to treat chest infections (Watt and Breyer-Brandwijk, 1962). The results obtained correlate somewhat with the traditional use. The combination against *S. pyogenes* related infections warrants caution as the possibility of antagonism is evident. In comparison with the dichloromethane: methanol extracts, the results obtained in the study for the triple combination shows that although the traditional use is through an infusion, tinctures may be a better option to treat respiratory infections.

8.2.3 *A. afra, Z. capense* and *Allium sativum* in combination

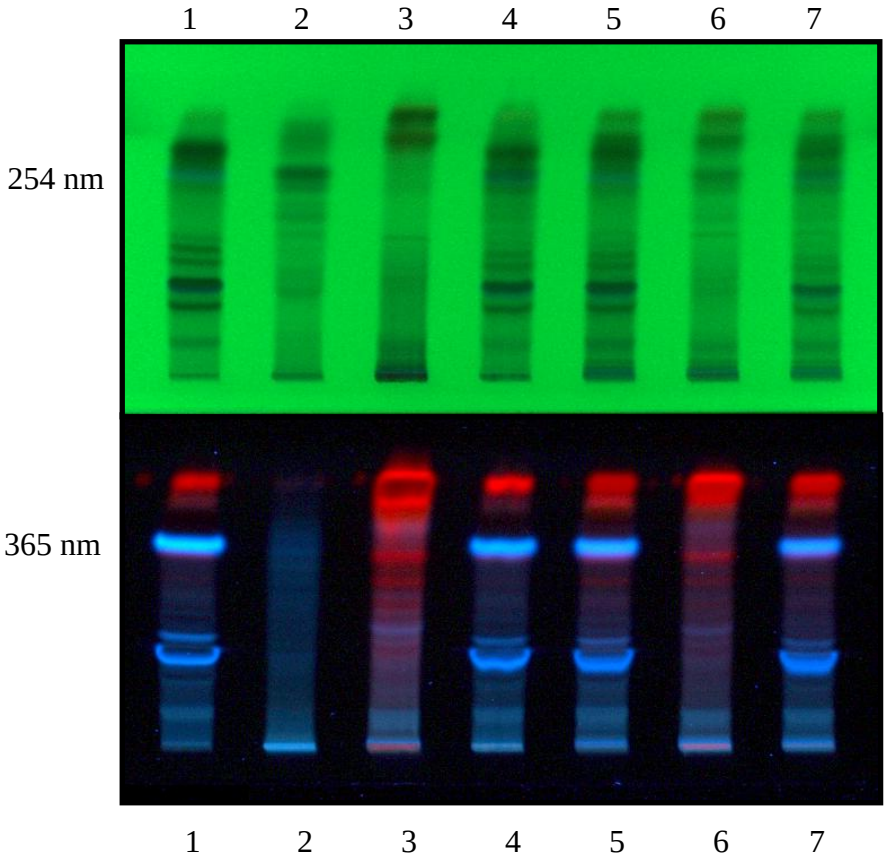
Garlic (*Allium sativum)* has been described and proven as a "cure all" (Harris et al., 2001). The Xhosa of South Africa drink a decoction of the leaf and the bulb of garlic as a febrifuge. This is prepared with the addition of *Z. capense* and *A. afra* (Watt and Breyer-Brandwijk, 1962). Hence, as the focus of the study was combinations with *A. afra*, this combination was studied. Garlic has been used for centuries in different cultures and societies (Rivlin, 2001). The antimicrobial activity has been extensively investigated (Sivam et al., 1997; Phay et al., 1999; Hsieh et al., 2001; Ward et al., 2002; Benkeblia, 2004). In Nigeria, it is used to treat abdominal discomfort, diarrhoea, otitis media and respiratory tract infections (Ankri and Mirelman, 1999; Jaber and Al-Mossawi, 2007; Abubakar, 2009).

The botanical description, locality, medicinal uses and phytochemistry of *A. sativum*, *A. afra* (Chapter 3) and *Z. capense* (Chapter 7) are discussed in Appendix A2, A3 and A9 respectively. The dichloromethane: methanol extracts of *A. afra*, *Z. capense* and *A. sativum* were evaluated using thin layer chromatography. MIC determination and ∑FIC interpretation was carried out on the, dichloromethane: methanol and the aqueous extracts according to the methods described in Chapter 2.

8.2.3.1 Chromatographic techniques

8.2.3.1.1 Thin layer chromatography

A large number of compounds were seen under the different wavelengths for each individual plant sample and majority of these were seen in the combination samples. In the combination of all three it is evident that majority of the same bands that appear in the individual TLC's are present in the combination. At R_f = 0.6, a major compound in *A. sativum* was found. At 365 nm, *A. sativum* shows pooling of the compounds at the bottom of the plate. The major green coloured compound found in *Z. capense* is seen in the combination with *A. afra* (Figure 8.4, lane 5), *A. sativum* (Figure 8.4, lane 6) and in the triple combination (Figure 8.4, lane 7) at R_f = 0.85.



1	*A. afra*
2	*A. sativum*
3	*Z. capense*
4	*A. afra* with *A. sativum*
5	*A. afra* with *Z. capense*
6	*A. sativum* with *Z. capense*
7	*A. afra*, *A. sativum* with *Z. capense*

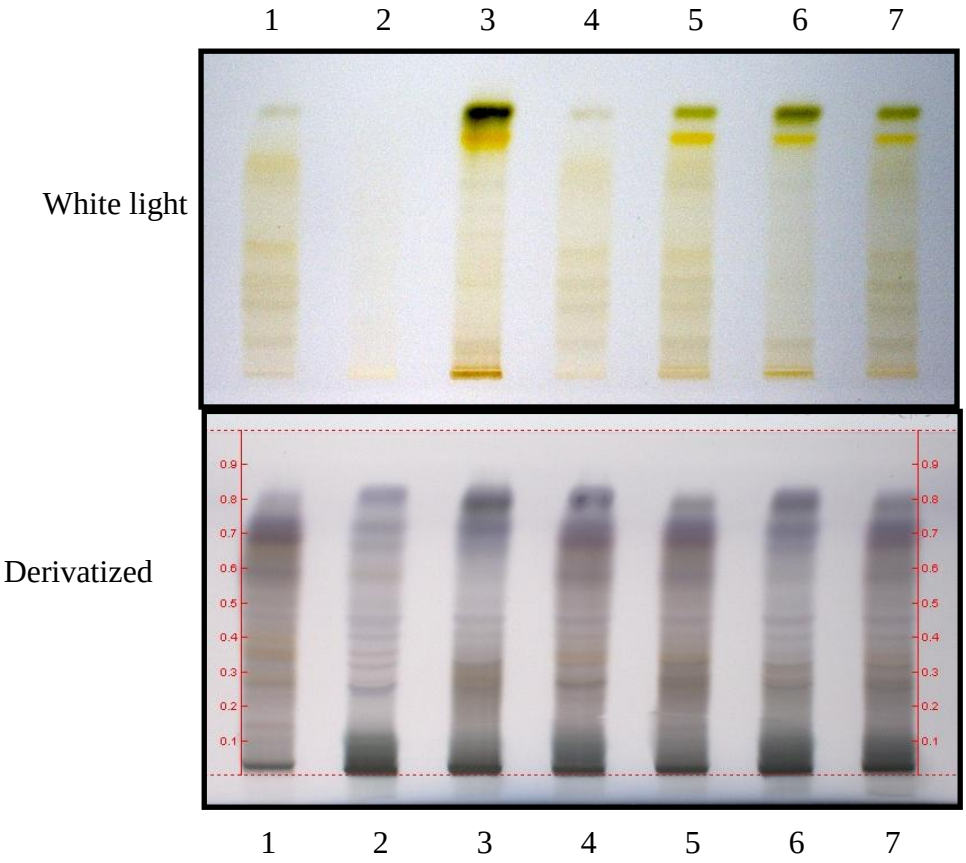


Figure 8.4 TLC plates of the dichloromethane: methanol extracts of *A. afra*, *Z. capense* with *A. sativum* alone and in combination at 254 nm, 365 nm, white light and visualized with vanillin-sulphuric acid reagent.

The combination of *A. afra*, *Z. capense* and *A. sativum* shows at 254 nm, 365 nm, under white light and after derivatization, the complexity of the mixture and the presence of compounds from each individual plant in the combination.

8.2.3.2 Antimicrobial analysis

8.2.3.2.1 MIC assays and FIC determination

8.2.3.2.1.1 Dichloromethane: methanol extracts

As shown in Table 8.6, *A. sativum* dichloromethane: methanol extracts generally showed poor to no activity at the highest concentration tested. The highest antimicrobial activity for *A. sativum* was against *C. neoformans*, which gave an MIC value of 2.0 mg/ml.

The combination of *A. afra* with *A. sativum* showed moderate activity against *M. catarrhalis* (MIC value = 1.0 mg/ml) and *S. pyogenes* (MIC value = 1.5 mg/ml). Some antimicrobial activity was noted against *C. neoformans*, *S. pneumoniae* and *M. smegmatis*.

Table 8.6 MIC and ∑FIC values of the dichloromethane: methanol extracts of *A. afra, Z. capense,* and *A. sativum* alone and in combination.

Micro-organism	MIC (mg/ml)/ ∑FIC value indicated in brackets where applicable							∑FIC	
	A. afra	***Z. capense***	***A. afra* with *Z. capense***	***A. sativum***	***A. afra* with *A. sativum***	***Z. capense* with *A. sativum***	***A. afra, Z. capense* with *A. sativum***	***A. afra, Z. capense* with *A. sativum***	**Interpretation**
K. pneumoniae NCTC 9633	6.3	1.7	2.0 (0.8)	≥16.0	8.0 (ND[1])	4.0 (ND[1])	6.0	ND [1]	Indifferent[2]
M. catarrhalis ATCC 23246	4.7	2.0	2.0 (0.7)	≥16.0	1.0 (ND[1])	8.0 (ND[1])	1.8	ND[1]	Indifferent[2]
E. faecalis ATCC 29212	6.3	1.3	2.0 (0.9)	≥16.0	6.0 (ND[1])	12.0 (ND[1])	3.0	ND[1]	Indifferent[2]
C. neoformans ATCC 90112	1.2	0.4	0.4 (0.7)	2.0	2.0 (1.3)	**0.8** (1.2)	1.2	1.5	Indifferent
S. pneumoniae ATCC 49619	0.5	1.5	0.3 (0.4)	8.0	2.0 (2.1)	4.0 (1.6)	1.5	1.4	Indifferent
S. pyogenes ATCC 8668	1.1	2.0	1.0 (0.7)	16.0	1.5 (0.7)	4.0 (1.1)	2.0	1.0	Additive
S. agalactiae ATCC 55618	3.0	4.0	4.0 (1.2)	16.0	≥16.0 (ND[1])	16.0 (2.5)	10.7	2.3	Indifferent
M. smegmatis (clinical)	1.7	0.8	2.0 (1.9)	≥16.0	4.0 (ND[1])	1.5 (ND[1])	0.5	ND[1]	Additive/Synergy[2]

*Values in bold indicate noteworthy activity.
ND[1] No ∑FIC value could be calculated as no MIC end point for *A. sativum*/combination was obtained at the highest concentration tested.
[2] Tentative interpretation according to MIC data.
Shaded area: results previously discussed in Chapter 3 and 7 but included here for reference purposes.
Controls excluded for the sake of brevity. Please refer to Chapter 3 for further detail regarding control values.

Poor to no activity was noted against *K. pneumoniae*, *E. faecalis* and *S. agalactiae*. ∑FIC values calculated showed additive interactions against *S. pyogenes* and indifference against *C. neoformans* and *S. pneumoniae*.

In the combination of the dichloromethane: methanol extracts of *Z. capense* with *A. sativum*, the best antimicrobial activity showing noteworthy moderate inhibition, was noted against *C. neoformans* with an MIC value of 0.8 mg/ml. Moderate inhibitory activity was seen against *M. smegmatis* with a MIC value of 1.5 mg/ml. Very poor activity was noted against *K. pneumoniae, M. catarrhalis, S. pneumoniae, S. pyogenes, E. faecalis* and *S. agalactiae*.

Indifferent interactions were calculated against *C. neoformans, S. pneumoniae, S. pyogenes* and *S. agalactiae* while tentative interpretations of indifference were made against *K. pneumoniae, M. catarrhalis, E. faecalis* and *M. smegmatis*.

For the triple combination i.e. *A. afra, Z. capense* with *A. sativum* dichloromethane: methanol extracts (Table 8.6), noteworthy strong inhibition was noted against *M. smegmatis* with a MIC value of 0.5 mg/ml. Moderate inhibition was noted against *C. neoformans* and *S. pneumoniae*. Some activity was obtained against *K. pneumoniae, M. catarrhalis, E. faecalis* and *S. pyogenes*. Very poor activity was seen against *S. agalactiae*. ∑FIC values calculated for the triple combination gave an additive interaction against *S. pyogenes*.

Tentative ∑FIC interpretations showed indifferent interactions against *K. pneumoniae, M. catarrhalis* and *E. faecalis* and synergistic or additive interactions against *M. smegmatis*.

The combination of *A. afra* with *Z. capense* dichloromethane: methanol extracts shows the interactions to be mostly additive, with one interaction displaying synergism (*S. pneumoniae*). For the combination of *A. afra* with *A. sativum*, only three interactions could be calculated, wherein two pathogens showed indifference (*C. neoformans* and *S. pneumoniae*) and one was additive (*S. pyogenes*). Tentative interpretations were made for the rest of the five pathogens. Two pathogens showed additive or indifferent interactions (*E. faecalis* and *M. smegmatis*), one pathogen was either indifferent or antagonistic (*S. agalactiae*), one was additive (*K. pneumoniae*) and one pathogen displayed synergy (*M. catarrhalis*). In the combination of *Z. capense* with *A. sativum*, mostly indifference was observed. The triple combination displayed varied interactions but mostly indifference. The results indicate that

the administration of a triple combination instead of any of the double combinations would have no benefit with respect to increased antimicrobial activity.

8.2.3.2.1.2 Aqueous extracts

Table 8.7 shows the antimicrobial activity of the aqueous extracts of *A. afra, Z. capense,* and *A. sativum* independently and in various combinations with each other. *A. sativum* aqueous extract showed no activity at the highest concentration tested against *K. pneumoniae, M. catarrhalis, E. faecalis, S. pneumoniae, S. agalactiae* and *M. smegmatis*. However, against *S. pyogenes*, very poor activity with a MIC value of 12.0 mg/ml was obtained. Moderate inhibition was found against *C. neoformans* and the MIC value obtained was 1.0 mg/ml.

The aqueous garlic extract was tested by Farbman et al. (1993) using the macro-dilution MIC assay. Results showed that *S. pneumoniae* was sensitive to a 1:2 dilution. It was ascertained that garlic had a broad spectrum of activity.

Jaber and Al-Mossawi (2007) tested the water, ethanol, dichloromethane, n-hexane extracts and the raw juice of garlic against different strains of *S. aureus* and *E. coli, Salmonella goldcoast* and *Klebsiella aerogenes*. Disc diffusion and chequerboard assays we performed on the water extract and the raw juice only as they produced the best results in the preliminary screening. However, poor activities were noted with the MIC values when they were tested in water and when they were subjected to different pH conditions (acid or alkali).

The water extracts did however, perform better that the solvent extracts even though poor activities were noted.

Abubakar (2009) tested the ethanol, chloroform and the aqueous extracts of *A. sativum* using the well diffusion method against *S. aureus, E. coli, S. pneumoniae* and *P. aeruginosa*. Results obtained against *S. pneumoniae* were 21 mm for the ethanol extracts, 19 mm for the chloroform extracts and 23 mm for the aqueous extracts. The aqueous extract was more active than the solvent or organic extracts, which is congruent with Roy et al. (2006), Jaber, and Al-Mossawi (2007).

Table 8.7 MIC and ∑FIC values of the aqueous extracts of *A. afra*, *Z. capense*, and *A. sativum* alone and in combination.

Micro-organism	MIC (mg/ml)/ ∑FIC value indicated in brackets where applicable							∑FIC	
	A. afra	***Z. capense***	***A. afra* with *Z. capense***	***A. sativum***	***A. afra* with *A. sativum***	***Z. capense* with *A. sativum***	***A. afra, Z. capense* with *A. sativum***	***A. afra, Z. capense* with *A. sativum***	**Interpretation**
K. pneumoniae NCTC 9633	12.0	13.3	12.0 (1.0)	≥16.0	16.0 (ND[1])	8.0 (ND[1])	16.0	ND[1]	Additive/Indifference[2]
M. catarrhalis ATCC 23246	8.0	16.0	8.0 (0.8)	≥16.0	16.0 (ND[1])	12.0 (ND[1])	12.0	ND[1]	Indifference[2]
E. faecalis ATCC 29212	7.0	16.0	≥16.0 (ND[1])	≥16.0	≥16.0 (ND[1])	12.0 (ND[1])	4.0	ND[1]	Synergy[2]
C. neoformans ATCC 90112	4.0	2.0	4.0 (1.5)	1.0	1.5 (0.9)	4.0 (3.0)	1.0	0.6	Additive
S. pneumoniae ATCC 49619	8.0	≥16.0	≥16.0 (ND[1])	≥16.0	≥16.0 (ND[1])	2.0 (ND[1])	≥16.0	ND[1]	Indifference/Antagonism[2]
S. pyogenes ATCC 8668	5.3	≥16.0	≥16.0 (ND[1])	12.0	16.0 (2.2)	4.0 (ND[1])	≥16.0	ND[1]	Indifference/Antagonism[2]
S. agalactiae ATCC 55618	1.7	0.4	1.3 (2.0)	≥16.0	3.0 (ND[1])	≥16.0 (ND[1])	1.0	ND[1]	Additive/Indifference[2]
M. smegmatis (clinical)	8.0	≥16.0	3.0 (ND[1])	≥16.0	8.0 (ND[1])	1.0 (ND[1])	1.0	ND[1]	Synergy/Additive[2]

Values in bold indicate noteworthy activity.
ND[1] No ∑FIC value could be calculated as no MIC end point for *A. sativum*/combination was obtained at the highest concentration tested.
[2] Tentative interpretation according to MIC data.
Shaded area: results previously discussed in Chapter 3 and 7 but included here for reference purposes.
Controls excluded for the sake of brevity. Please refer to Chapter 3 for further detail regarding control values.

When *A. afra* was combined with *A. sativum* aqueous extracts, MIC values were not obtained against *E. faecalis* and *S. pneumoniae* at the highest concentration tested. Very poor antimicrobial activity was seen against *K. pneumoniae*, *M. catarrhalis*, *S. pyogenes* and *M. smegmatis*. Some activity was noted against *S. agalactiae* with a MIC value of 3.0 mg/ml. Moderate inhibitory activity (MIC value of 1.5 mg/ml) was obtained against *C. neoformans*. When the ∑FIC values were calculated, additive interactions were noted against *C. neoformans* and indifferent activity was seen against *S. pyogenes*.

When *Z. capense* was combined with *A. sativum*, MIC values were varied from 1.0-12.0 mg/ml. Very poor activity was noted against *K. pneumoniae*, *M. catarrhalis*, and *E. faecalis*. Some activity was seen against *C. neoformans*, *S. pneumoniae* and *S. pyogenes*. The best activity (MIC value = 1.0 mg/ml) found for the combination was moderate inhibitory activity and it was obtained against *M. smegmatis*. ∑FIC values for the combination of *A. sativum* with *Z. capense* showed an indifferent interaction against *C. neoformans*.

For the combination of the three plants i.e. *A. afra*, *A. sativum* and *Z. capense*, no activity was seen against *S. pneumoniae* and *S. pyogenes* while very poor activity was noted against *K. pneumoniae* and *M. catarrhalis*. Some antimicrobial activity was noted against *E. faecalis* with a MIC value of 4.0 mg/ml. The best activity of the triple combination was found against C. *neoformans* as well as against *S. agalactiae* and *M. smegmatis* with moderate inhibitory activity (MIC values of 1.0 mg/ml). A ∑FIC value of 0.6 and denoting an additive interaction were obtained for the combination of the three plants against *C. neoformans*. Tentative interpretations of the MIC data on the interactions of the combination showed additive or indifferent interactions against *K. pneumoniae* and *S. agalactiae*. An indifferent interaction was noted against *M. catarrhalis* and indifference or antagonistic interactions against *S. pneumoniae* and *S. pyogenes* was seen. Synergy was evident against *E. faecalis* and additivity or synergy was noted against *M. smegmatis*.

The combination of *A. afra* with *Z. capense* (aqueous extracts) showed the interactions to be varied depending on the pathogen studied. The combination of *A. afra* with *A. sativum* showed additivity against one pathogen (*C. neoformans*) and an indifferent interaction against another (*S. pyogenes*). The combination of *A. sativum* with *Z. capense* resulted in interactions against three pathogens (*S. pneumoniae*, *S. pyogenes* and *M. smegmatis*) as synergy. The triple combination showed only one definite additive interaction against *C. neoformans*

therefore the triple combination could be advantages in *E. faecalis* related infections. However, care should be taken against *S. pneumoniae* and *S. pyogenes* as the possibility of antagonism exists.

In light of these interactions, the results do not show any significant enhancement of antimicrobial activity with the triple combination. Thus, the addition of the third plant by traditional healers could possibly due to the presence of other pharmacological properties that may assist in holistic healing. These properties could contribute to the treatment of the respiratory diseases by producing anti-inflammatory, anti-histamine, decongestant, anti-pyretic or analgesic effects.

8.3 Conclusions

- The TLC results of the combination of *A. afra* with *O. asteriscoides* and *E. globulus*; the combination of *A. afra, E. globulus* and *L. randii*; and the combination of *A. afra* with *Z. capense* and *A. sativum* highlighted the complexity of the plants being tested individually and in combination.
- The MIC values of the combination of *A. afra* with *O. asteriscoides* and *E. globulus* essential oils showed noteworthy antimicrobial activity against *S. pneumoniae, S. pyogenes* and *M. smegmatis*. Antagonism was seen when the ∑FIC values were determined against *C. neoformans*. Additivity was also evident against *K. pneumoniae, M. catarrhalis, E. faecalis* and *S. agalactiae.*
- The MIC values of the dichloromethane: methanol extracts of the combination of *A. afra* with *O. asteriscoides* and *E. globulus* showed noteworthy activity against *C. neoformans, S. pneumoniae* and *S. agalactiae*. Synergistic activity was noted when the ∑FIC values were calculated against *K. pneumoniae* and *S. agalactiae*. Antagonism was noted against *M. catarrhalis* and *E. faecalis*.
- Very good antimicrobial activity was noted for the combination of *A. afra* with *E. globulus* and *L. randii* dichloromethane: methanol extracts with noteworthy MIC values against all the pathogens tested with the exception of *S. agalactiae*. The ∑FIC values of the triple combination showed predominantly additive and synergistic activity against all the pathogens tested, except for the indifference noted against *S. agalactiae*.

- The aqueous extracts of the combination of *A. afra* with *E. globulus* and *L. randii* showed some antimicrobial activity against *K. pneumoniae*, *E. faecalis*, *C. neoformans*, *S. agalactiae* and *M. smegmatis*. Additive interactions were calculated against *K. pneumoniae*.
- The dichloromethane: methanol extracts of the combination of *A. afra with A. sativum* and *Z. capense* showed noteworthy inhibitory activity against *M. smegmatis*.
- Moderate inhibition was seen in the MIC values, of the aqueous extracts of the combination of *A. afra* with *A. sativum* and *Z. capense*, obtained against *C. neoformans*, *S. agalactiae* and *M. smegmatis*. Indifference was predominant in the interactions of the combination with synergy noted against *E. faecalis*. Synergy or additive interactions were noted against *M. smegmatis*.

Chapter 9

Artemisia afra in combination with adjuncts

9.1Introduction

An adjunct is added as a supplementary agent to an active drug and is not an essential part of the formulation (http://www.encyclopedia.com/topic/adjunct.aspx). With respect to pharmacological applications, an example would be the use of syrup (sugar) to mask the taste of paracetamol in Panado®. The addition of the green colour and flavouring is also an example of the use of an adjunct. In this case, it is added to provide flavour and is more aesthetically pleasing to children.

The use of adjuncts with medicinal plants by traditional healers is a popular, yet unstudied area in phytomedicine (Watt and Breyer-Brandwijk, 1962; Hutchings et al., 1996; van Wyk et al., 2008). Investigation of traditional medicinal practices have revealed a number of instances where plants are mixed with milk, honey, salt, butter, and yoghurt to improve the acceptability of the medicine (Roberts, 1990; Hutchings et al., 1996; Jäger, 2003; Johnston, 2009; Thring and Weitz, 2006; van Wyk et al., 2008; van Wyk, 2008b; Liu, et al., 2009; Poonam and Singh, 2009). A combination of plants are frequently used and sometimes mixed with adjuncts before administration, the most common being honey, milk, sugar, salt, fat and brandy (Figure 9.1).

Traditional preparations often require sugar or honey to make syrups (van Wyk et al., 2008). The use of these provides a sweet vehicle for the medicament, allowing palatability of the plant and thus making the patient more comfortable with swallowing it. Fat or oil are also used as vehicles, perhaps to increase viscosity for topical applications or to provide an oily vehicle for eardrops (Thring and Weitz, 2006; van Wyk, 2008b). They are readily available and the familiarity of these vehicles provides a level of comfort when being used by the patients. Sometimes milk is used instead of water when making decoctions or teas perhaps because of the availability or even because a particular plant may sometimes be more soluble in milk than water (Bhattarai et al., 2006). Brandy and vinegar are used to make tinctures (Watt and Breyer-Brandwijk, 1962). They are also commonly used when preparing poultices for wounds.

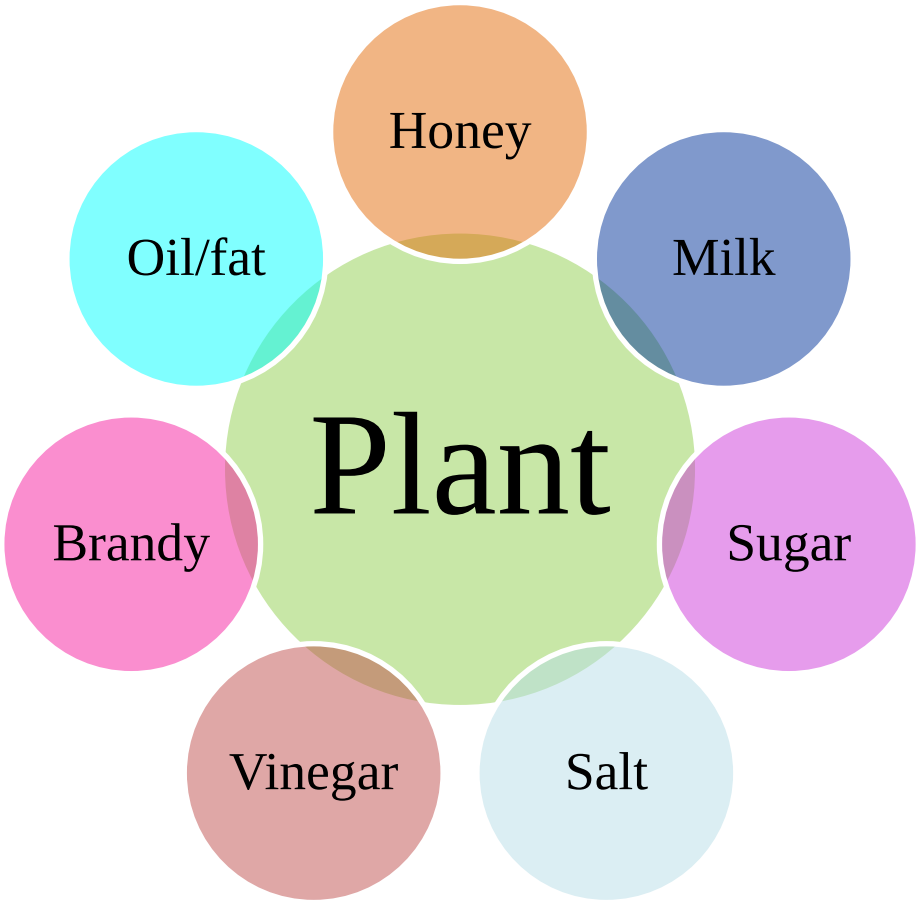


Figure 9.1 The various adjuncts used in traditional medicinal practices. (Roberts, 1990; Hutchings et al., 1996; Jäger, 2003; Johnston, 2009; Thring and Weitz, 2006; van Wyk et al., 2008; van Wyk, 2008b; Liu, et al., 2009).

One example where the practical use of adjuncts is applied is the use of the leaf and stem of *Lippia javanica* as a weak tea made with either water or milk for coughs, colds and bronchial complaints (van Wyk et al., 2009; Watt and Breyer-Brandwijk, 1962). Another example is the use of *A. afra* with honey or sugar for coughs and colds (Roberts, 1990; Liu et al., 2009). It is also commonly used with brandy for infantile colic (Watt and Breyer-Brandwijk, 1962). These and other examples are elaborated on in Table 9.1.

Aside from the practical applications of these adjuncts i.e. to aid in medicinal plant delivery, it is possible that each adjunct could have a therapeutic effect. There are many instances where these adjuncts are used independently as remedies. There are documented incidents where vinegar and honey have been used for wound care from the time of Hippocrates (Johnston, 2009). In fact, evidence suggests that vinegar on its own has therapeutic value, in the management of blood glucose, as suggested by Johnston (2009). In order to accurately assess the activity and effectiveness of the plants, it is important to take into account the

traditional preparation of the medicine (Jäger, 2003). The addition of honey or sugar could prove to have an effect on the extent to which that plant works.

Table 9.1 Various plants used in combination with adjuncts.

Plant/s	Adjunct	Use	Complaint	Reference
A. afra with *Tetradenia riparia*	Salt	Decoction	Coughs	Hutchings et al. (1996).
A. afra with *Warburgia salutaris*	Honey, brown sugar	Infusion	Cough	Felhaber, (1997).
Agathosma betulina	Vinegar, brandy	Soaked leaves, poultice, sprayed	Wounds, sprains, contusions	Thring and Weitz, (2006); van Wyk, (2008b).
A. afra	Honey, sugar	Infusion, decoction	Bronchial troubles, coughs and colds, chills, dyspepsia, loss of appetite, stomach ache, colic, croup, whooping cough, gout and as a purgative.	Roberts, (1990); Liu et al. (2009).
A. afra	Brandy, vinegar	Moistened leaf	Infantile colic	Watt and Breyer-Brandwijk, (1962).
A. afra	Brandy	Tincture taken by mouth	Colic	Watt and Breyer-Brandwijk, (1962).
A. afra	Milk	Suspension of ground up plant as enema	Constipation and intestinal worms	Watt and Breyer-Brandwijk, (1962).
Ballota africana	Sugar	Syrup	Colds	Thring and Weitz, (2006).
Carbobrotus edulis or *C. acinaciformis* or *C. murii*	Milk	Added to the squeezed juice of leaves	Stomach troubles	Thring and Weitz, (2006).
Cissampelos capensis	Vinegar, sugar	Root infusion	Fever	van Wyk et al. (2008b).
Clematis brachiata	Honey	Decoction	Sinusitis	Felhaber, (1997).
Crassula ovata	Milk	Boil leaves	Diarrhoea	van Wyk, (2008b).
Crassula tetragona	Milk	Boiled leaves	Diarrhoea	van Wyk, (2008b).
Cynodon dactylon	Fat	Rubbed in skin	Gout	van Wyk, (2008b).
Datura spp.	Potassium nitrate	Powdered	Asthma	van Wyk, (2008b).

Plant/s	Adjunct	Use	Complaint	Reference
Eberlanzia spinosa	Sugar	Decoction of leaves	Angina	van Wyk et al. (2008).
Elytropappus rhinocerotis	Brandy, vinegar	Leaves are soaked and taken, Soaked leaves on a cloth, wrapped around sore area	Bladder, kidney, convulsions, diabetes, fever, headache, cough, cold, flu, stomach-ache	Thring and Weitz, (2006).
Euryops multifidus	Fat	Resins mixed with fats, applied	Sores	van Wyk, (2008b).
Exomis microphylla/ Exomis axyroides	Milk	Leaf decoction	Khoi remedy for epilepsy; winds, cramps and convulsions in infants	van Wyk, (2008b).
Galenia africana	Salt	Boil in water and wash wounds	Wounds	van Wyk et al. (2008).
Galium tomentosum	Sugar	Infusion of the roots	Remove acid in babies	van Wyk et al. (2008).
Gnidia polycephala	Milk	Drink a root infusion mixed with milk, purgative	Constipation	van Wyk et al. (2008).
Heamanthus coccineus	Vinegar	Sliced bulbs	Expectorant, diuretic	van Wyk, (2008b).
Heeria argentea	Sweet oil	Gum and oil mixed to make a plaster	Burns. Wounds and tender nipples	van Wyk, (2008b).
Helichrysum litorale	Fat	Powdered herb, applied	Ulcers	van Wyk, (2008b).
Lippia javanica	Milk	Infusion of leaf and stem	Coughs, colds, bronchial troubles	Watt and Breyer-Brandwijk, (1962).
Melianthus comosus	Vinegar	Apply fresh leaves as a poultice	Knee pain	van Wyk et al. (2008).
Pelargonium antidysenterium	Milk	Decoction	Dysentery	van Wyk, (2008b).
Peliostomum origanoides	Sweet oil/linseed oil or cod liver oil	Tea made from the twigs + oil	Acid in babies	van Wyk et al. (2008).

Plant/s	Adjunct	Use	Complaint	Reference
Pteronia onobromoides	Fat	Mixed with powdered leaf	Burns, sunburn, earache	van Wyk, (2008b).
Schinus molle	Vinegar	Place fresh leaves in a cloth, with vinegar and apply to the head	Headache	van Wyk et al. (2008).
Sutherlandia microphylla	Honey	Drink	General medicine	van Wyk et al. (2008).
Trichilia emetica	Milk, honey	Mixed with powdered roots	Asthma, cough, bronchial trouble, fever, vomiting	Sutovska et al. (2009).
Tulbaghia alliacea	milk	Infusion	Intestinal worms, fever, influenza high blood pressure tuberculosis	van Wyk, (2008b).
Tulbaghia violecea	Milk	Infusion	Intestinal worms, fever, influenza, high blood pressure, TB	van Wyk, (2008b).
Tulbaghia violecea	Castor oil	Ear drops	Earache	Thring and Weitz, (2006).
Tulbaghia violecea	Brandy	Seed-pods soaked and taken	Sleeplessness, heart condition, relieves stomach pain, convulsions	Thring and Weitz, (2006).
Viscum capense	Brandy	Whole pieces soaked and taken	Fever, cold symptoms	Thring and Weitz, (2006).

Some of these adjuncts e.g. honey and vinegar has shown independent antimicrobial activity (Wahdan, 1998; Johnston, 2009). In fact, as alternate medicine increases in popularity, many studies are aiming to verify and support the use of these traditional remedies. For a number of years the interest in the benefits of honey has increased. The antimicrobial activity of honey alone, at various concentrations, has been established. These are for bacteria commonly found to infect wounds, as well as a number of fungi (Efem et al., 1992; Molan, 1996; Malika et al., 2005; Alandejani et al., 2009). These studies further provide an interest in the possible contribution of honey and other adjuncts to the efficacy of traditional plant medicines.

This study was undertaken with the aim of finding a scientific rationale for the use of adjuncts in combination with medicinal plants that are commonly used in South African traditional medicine. As the primary focus of this study, *A. afra* was chosen as the main plant around which adjunct studies has focussed. This plant was chosen due to its prominence as

one of the foremost plants used frequently in combinations with other plants as well as with adjuncts. This is shown Table 1, where *A. afra* was commonly found to be combined with honey, salt, brandy, and milk (Watt and Breyer-Brandwijk, 1962).

9.2 Results and discussion

9.2.1 Individual MIC values

Individual MIC values of adjuncts show no antimicrobial activity against *M. catarrhalis*, *E. faecalis*, *K. pneumoniae* and *C. neoformans*. Exceptions were noted with honey where very low activity was seen against *K. pneumoniae*. Vinegar was also poorly active against *K. pneumoniae* and *C. neoformans* with MIC values of 16.0 mg/ml. Table 9.2 documents the individual MIC values for all adjuncts.

Table 9.2 Individual mean MIC values of all adjunct samples tested.

Sample	**MIC values (mg/ml)**			
	***M. catarrhalis* ATCC 23246**	***E. faecalis* ATCC 29212**	***K. pneumoniae* NCTC 9633**	***C. neoformans* ATCC 90112**
Honey	≥16.0	≥16.0	8.0	≥16.0
Orange Blossom Honey (100%)	≥25.0	≥25.0	≥25.0	≥25.0
Wild Blossom Honey (100%)	≥25.0	≥25.0	≥25.0	≥25.0
Blue Gum Honey (100%)	≥25.0	≥25.0	≥25.0	≥25.0
Salt (4%)	≥10.0	≥10.0	≥10.0	≥10.0
Skim milk	≥25.0	≥25.0	≥25.0	≥25.0
Full cream milk	≥25.0	≥25.0	≥25.0	≥25.0
Brandy	≥16.0	≥16.0	≥16.0	≥16.0
Vinegar	≥16.0	≥16.0	16.0	16.0

9.2.2 Honey in combination with *A. afra*

The different types of honey used in this study were commercially purchased orange blossom honey, wild blossom honey and blue gum honey.

The testing of different honey samples was carried out to determine if there is any differing microbial activity among differing types of honey. Studies conducted on honey samples in the past have shown that honey from different sources may display different activities (Lee et al., 2008). Honey (100%) was used as it displayed good antimicrobial activity in a previous study (Efem et al., 1992).

The MIC values represented in Table 9.3 are those of the different types of honey in combination with the essential oil and extracts of *A. afra.* Significant values to note are those of honey at 64.0 mg/ml with the *A. afra* aqueous extract, which shows considerably better MIC values than that of honey alone. Against *M. catarrhalis* and *E. faecalis*, some activity was obtained with a MIC of 3.0 mg/ml. Similarly, moderate inhibition was obtained against *K. pneumoniae* with a MIC of 1.5 mg/ml. For the 100% blue gum honey in combination with the dichloromethane: methanol extracts of *A. afra*, some activity was noted against *K. pneumoniae* and *C. neoformans*. *A. afra* aqueous extract in combination with 100% orange blossom or wild blossom showed some activity against *E. faecalis* and *K. pneumoniae*. Moderate inhibitory antimicrobial activity (1.3 mg/ml) was noted against *C. neoformans* in the combination of *A. afra* dichloromethane: methanol extract and 100% orange blossom honey.

Although MIC values can be interpreted as being favorable in the combinations tested, a better understanding of the interactions of the combinations can be sought by calculating of ∑FIC values. In the testing of the use of adjuncts in combination with *A. afra* most of the results of the MIC values showed no end point at the concentrations tested. Thus, bar graphs have been constructed to make comparisons of the ∑FIC interactions. Figure 9.2 shows the ∑FIC values of the combinations against the four different micro-organisms tested.

In the combination of *A. afra* and honey at a concentration of 64.0 mg/ml, the majority of combinations showed indifference. The combination of orange blossom honey at 64.0 mg/ml in combination with *A. afra* aqueous extracts against *C. neoformans* showed an additive interaction.

Table 9.3 MIC values for different types of honey in combination with *A. afra* samples.

Sample	MIC values (mg/ml)			
	M. catarrhalis **ATCC 23246**	***E. faecalis*** **ATCC 29212**	***K. pneumoniae*** **NCTC 9633**	***C. neoformans*** **ATCC 90112**
A. afra essential oil	8.0	8.0	8.0	13.0
A. afra dichloromethane: methanol extracts	4.0	4.0	2.0	4.3
A. afra aqueous extract	4.0	8.0	8.0	13.0
A. afra essential oil: honey (64.0 mg/ml)	16.0	16.0	16.0	48.0
A. afra essential oil: orange blossom honey (100%)	57.0	57.0	57.0	28.5
A. afra essential oil: wild blossom honey (100%)	57.0	57.0	57.0	14.3
A. afra essential oil: blue gum honey (100%)	57.0	57.0	57.0	14.3
A. afra dichloromethane: methanol extracts: honey (64.0 mg/ml)	8.0	8.0	8.0	22.0
A. afra dichloromethane: methanol extracts orange blossom honey (100%)	10.3	10.3	5.1	1.3
A. afra dichloromethane: methanol extracts wild blossom honey (100%)	10.3	10.3	5.1	2.6
A. afra dichloromethane: methanol extracts blue gum honey (100%)	10.3	5.1	2.6	2.5
A. afra aqueous extract: honey (64.0 mg/ml)	**3.0**	**3.0**	**1.5**	8.0

Sample	MIC values (mg/ml)			
	***M. catarrhalis* ATCC 23246**	***E. faecalis* ATCC 29212**	***K. pneumoniae* NCTC 9633**	***C. neoformans* ATCC 90112**
A. afra aqueous extract: orange blossom honey (100%)	26.0	5.1	2.6	41.0
A. afra aqueous extract: wild blossom honey (100%)	26.0	5.1	2.6	41.0
A. afra aqueous extract: blue gum honey (100%)	26.0	10.3	10.3	41.0

In the combination of *A. afra* essential oil and orange blossom honey at 100%, antagonism was observed. The dichloromethane: methanol extract showed synergistic activity against *C. neoformans*. The aqueous extracts showed additivity against *K. pneumoniae*, and antagonism against *E. faecalis* and *C. neoformans*.

These results show a high incidence of antagonism with essential oil and aqueous extract combinations of *A. afra* with honey. In traditional healing practices, essential oils are seldom combined with honey. Synergism seems to be restricted to the combinations where dichloromethane: methanol extracts are combined with all three 100% honey products against *C. neoformans*. Results obtained support the assumption that the addition of honey is mainly for sweetening the plant decoctions thereby disguising any unpleasant taste that may be encountered by the patient.

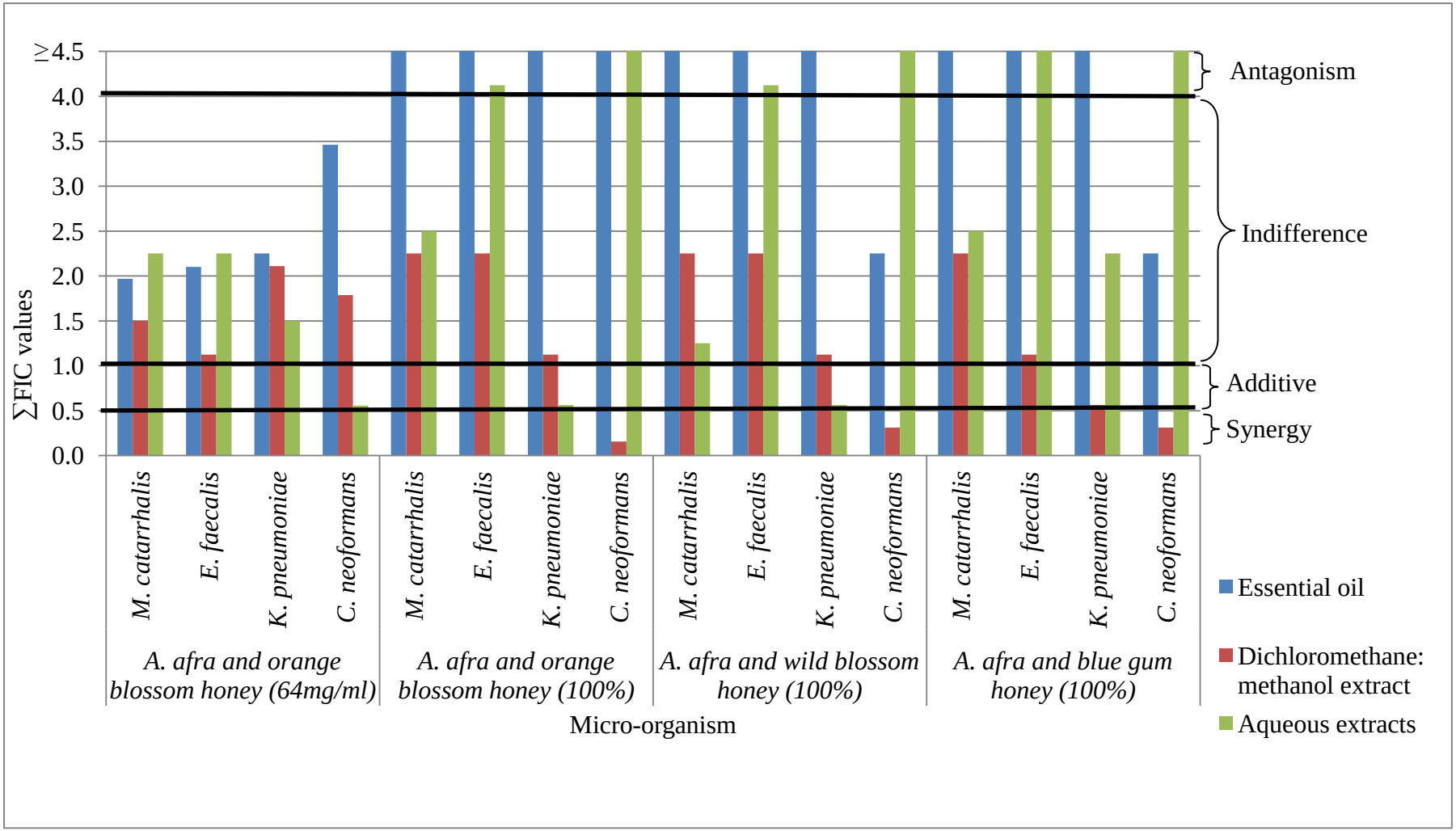


Figure 9.2 The ∑FIC interactions of the different types of honey in combination with *A. afra*.

9.2.3 Skim milk and full cream milk in combination with *A. afra*

The MIC values obtained for the combination of *A. afra* together with skim milk and full cream milk are shown in Table 9.4. Both these were tested as the traditional healer uses either one depending on circumstances.

MIC values in combination show poor activity. However, against *M. catarrhalis*, in the combination of *A. afra* aqueous extract and skim milk display some antimicrobial activity (MIC value of 5.1 mg/ml).

Table 9.4 MIC values of *A. afra* in combination with milk.

Sample	MIC values (mg/ml)			
	***M. catarrhalis* ATCC 23246**	***E. faecalis* ATCC 29212**	***K. pneumoniae* NCTC 9633**	***C. neoformans* ATCC 90112**
A. afra essential oil	8.0	8.0	8.0	13.0
A. afra dichloromethane: methanol extracts	4.0	4.0	2.0	4.3
A. afra aqueous extract	4.0	8.0	8.0	13.0
A. afra essential oil: skim milk	28.5	57.0	28.5	14.3
A. afra essential oil: full cream	57.0	28.5	28.5	41.5
A. afra dichloromethane: methanol extracts: skim milk	20.5	20.5	20.5	20.5
A. afra dichloromethane: methanol extracts: full cream milk	20.5	20.5	10.3	20.5
A. afra aqueous extract: skim milk	5.1	20.5	41.0	30.7
A. afra aqueous extract: full cream milk	20.5	≥41.0	41.0	41.0

Figure 9.3 shows the ∑FIC interaction of the combination of skim or full cream milk with *A. afra* essential oils, dichloromethane: methanol and aqueous extracts. The interactions with skim milk show mainly indifference. Exceptions were seen in the combination of *A. afra*

aqueous extract with skim milk where an additive interaction was noted against *M. catarrhalis*.

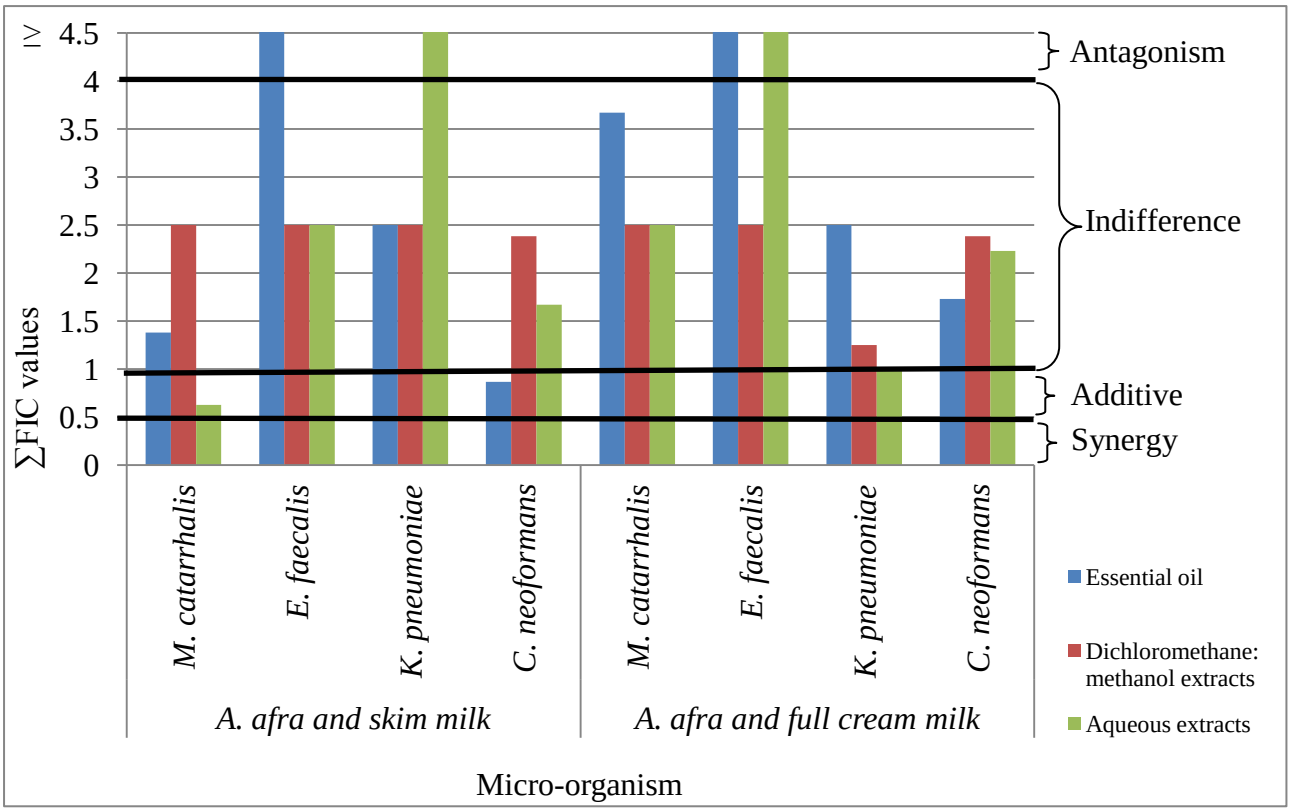


Figure 9.3 *A. afra* in combination with milk.

An additive interaction was also noted for the essential oil and skim milk combination against *C. neoformans*. Antagonism was seen against *E. faecalis* for the essential oil combination in both skim milk and full cream milk. Antagonism was also evident against *K. pneumoniae* and *E. faecalis* for the aqueous extract combination with skim milk and full cream milk respectively.

Essential oils are not known to be administered with milk and hence the antagonism noted with the essential oils may not be significant when observing traditional practises. Milk may possibly help alter the taste of the medicine and therefore make it more acceptable to the patient to consume. For further consideration, there is the possibility that the role of milk may be more beneficial as an excipient in the formulation to improve the absorption of the plant mixture. In addition, as milk has a soothing and calming connotation for many people it may also be employed as a means to increase comfort to the gastrointestinal tract of the patient.

With the exception of one or two instances, it is also noted that the addition of milk has no negative impact on the antimicrobial activity of *A. afra*.

9.2.4 Vinegar and/brandy in combination with *A. afra*

The concentration of the vinegar sample tested was 64.0 mg/ml. The MIC values obtained when vinegar was combined with *A. afra* essential oil showed very poor activities (Table 9.5).

Table 9.5 *A. afra* in combination with vinegar and brandy.

Sample	MIC values (mg/ml)			
	M. catarrhalis **ATCC 23246**	***E. faecalis*** **ATCC 29212**	***K. pneumoniae*** **NCTC 9633**	***C. neoformans*** **ATCC 90112**
A. afra essential oil	8.0	8.0	8.0	13.0
A. afra dichloromethane: methanol extract	4.0	4.0	2.0	4.3
A. afra aqueous Extract	4.0	8.0	8.0	13.0
A. afra essential oil: brandy	48.0	48.0	48.0	24.0
A. afra essential oil: vinegar	48.0	48.0	48.0	24.0
A. afra dichloromethane: methanol extract : brandy	4.0	16.0	16.0	32.0
A. afra dichloromethane: methanol extract : vinegar	16.0	8.0	8.0	16.0
A. afra aqueous extract: brandy	32.0	32.0	32.0	4.0
A. afra aqueous extract: vinegar	32.0	32.0	16.0	8.0

In combination with the dichloromethane: methanol extract, some activity was noted against *M. catarrhalis* with a MIC value of 4.0 mg/ml. Essential oil and aqueous extract combinations with vinegar have antagonistic interactions against the pathogens tested in this study. The ∑FIC values for all pathogens and in combination with essential oil and water

extracts show values >4.0 (Figure 9.4). The dichloromethane: methanol extract show additive interactions against *M. catarrhalis* and *E. faecalis*. All the *A. afra* combinations with vinegar showed indifference against *C. neoformans*.

One of the possible reasons for antagonistic actions could be that the main use of vinegar is in softening of the leaves for the treatment of colic. The testing of pathogens known to cause gastrointestinal distress may yield better interactive efficacies.

Brandy is one of the most commonly used adjuncts to medicinal plants. It is mainly used to soften and soak plant leaves and twigs. The brandy is also commonly used in preparations for wounds (Table 9.1).

The MIC values of brandy in combination with *A. afra* are shown in Table 9.5. Poor antimicrobial activities were noted in the essential oil, dichloromethane: methanol extract and the aqueous extract combinations against all four pathogens tested.

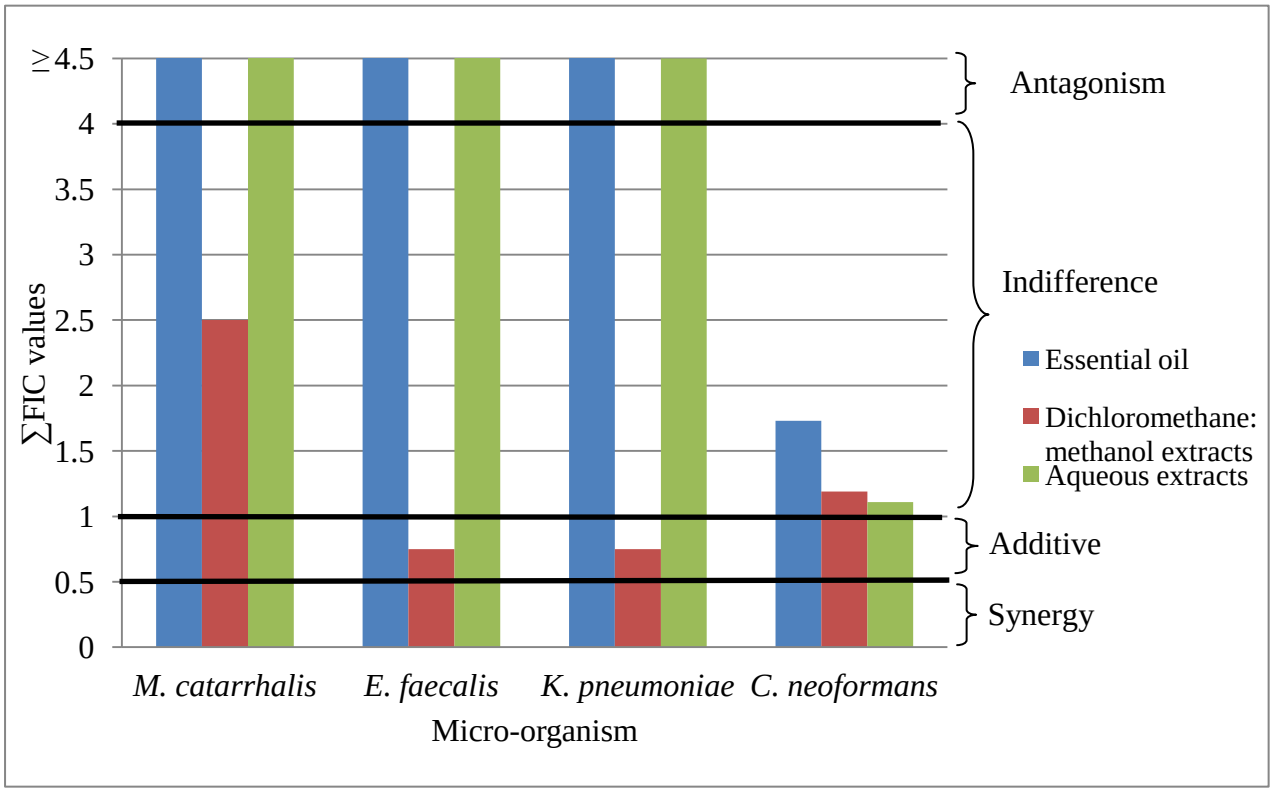


Figure 9.4 *A. afra* in combination with vinegar.

One exception with some activity (MIC value of 4.0 mg/ml) was obtained against *C. neoformans* for the aqueous extract combination of *A. afra* with brandy.

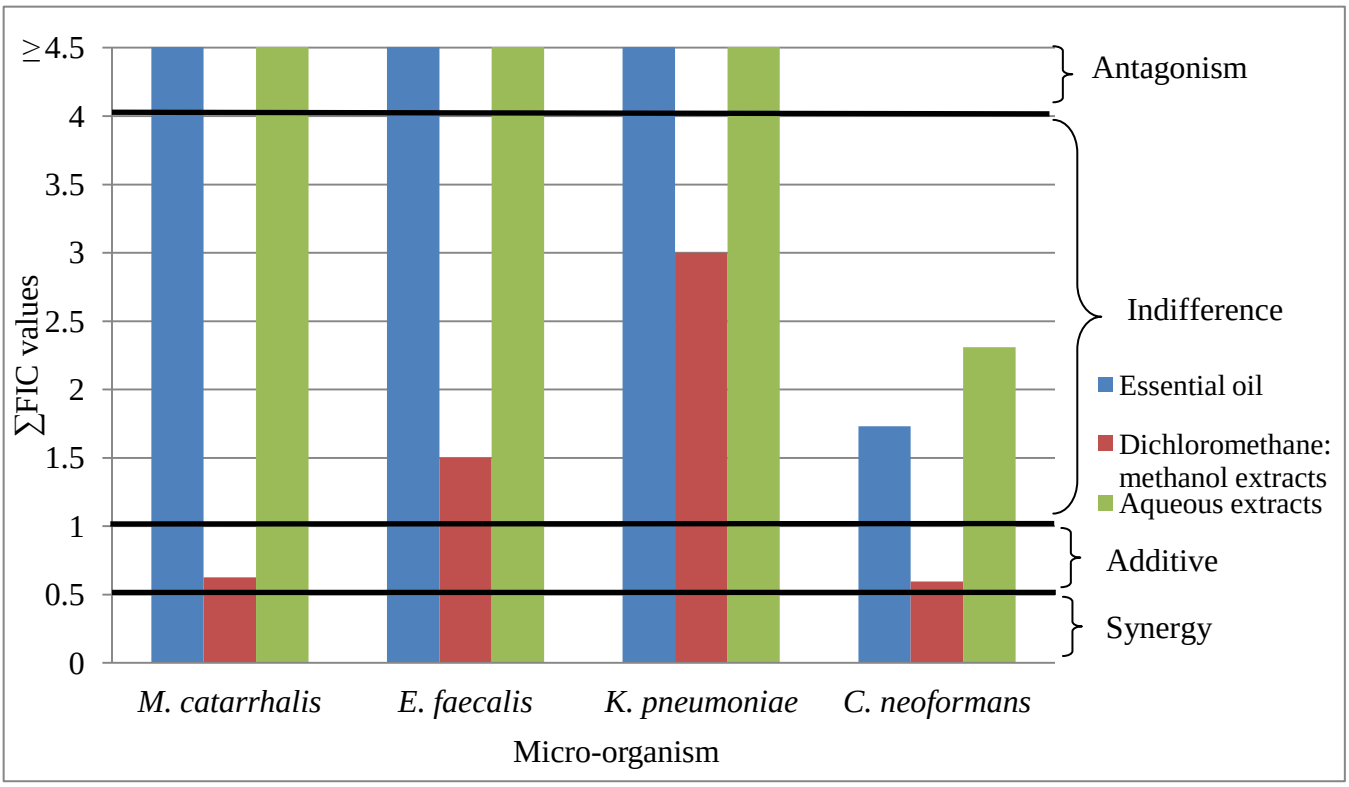


Figure 9.5 Brandy in combination with *A. afra*.

Brandy, in combination with the essential oil and water extract of *A. afra* show antagonistic interactions against three bacteria tested (Figure 9.5). This supports the common, modern medicinal practice of discouraging the imbibing of brandy and other alcoholic drinks with medication. The dichloromethane: methanol extract, on the other hand showed additive interactions against *M. catarrhalis* and *C. neoformans*. Indifference was noted against *E. faecalis* and *K. pneumoniae*. This provides some credibility to the most common use in the preparation of tinctures and poultices. Further studies, using other plants and bacteria specifically for wound healing are needed to explore this possibility.

9.2.5 Salt in combination with *A. afra*

Salt is considered as an antimicrobial agent as it works by restricting bacterial growth. This is done in two ways. Either by lowering the amount of free water molecules around the bacteria e.g. in food or body tissues. The salt water creates a hypertonic environment then causing water to rush out of the bacteria by osmosis and thus killing or rupturing the cells. Moisture is essential to bacterial growth and survival and without enough water, they do not grow well. Furthermore, bacteria do not thrive or grow well in salty environments. Salt restricts microbial growth by interfering with the microbe's enzyme activity and thus weakens its

DNA molecular structure (Parish, 2006; Shee et al., 2010). The study by Shee et al. (2010) tested the potential of salt and sugar as food preservatives. A 2.5% salt solution was used to test the preservative efficacy. Results demonstrated that the solution was able to contain the growth of bacteria tested (not specified) until the third day. The study by Milne et al. (2007) examined the effects of a 15% salt impregnated dressing for critically colonized wounds. Results showed a reduction of bacteria in critically colonized wounds in the majority of the patients following the 24 hrs after application.

The MIC values obtained for the combination of salt with *A. afra* essential oil, dichloromethane: methanol and aqueous extract are shown in Table 9.6. The MIC values show very poor activity against all the pathogens for the essential oils, dichloromethane: methanol and aqueous extracts.

The results of the interactions of *A. afra* and salt combinations are shown in Figure 9.6. All pathogens in combination with essential oil, aqueous extract, and dichloromethane: methanol extracts respond with indifference (∑FIC values calculated were between 1.0 and 4.0).

Table 9.6 MIC values of salt in combination with *A. afra.*

Sample	MIC values (mg/ml)			
	M. catarrhalis **ATCC 23246**	***E. faecalis*** **ATCC 29212**	***K. pneumoniae*** **NCTC 9633**	***C. neoformans*** **ATCC 90112**
A. afra essential oil	8.0	8.0	8.0	13.0
A. afra dichloromethane: methanol extracts	4.0	4.0	2.0	4.3
A. afra aqueous extract	4.0	8.0	8.0	13.0
A. afra essential oil: salt	21.0	21.0	18.5	21.0
A. afra dichloromethane: methanol extracts: salt	13.0	13.0	13.0	6.5
A. afra aqueous extract: salt	13.0	26.0	13.0	26.0

Although essential oil salt combination values are slightly lower than the other extracts it is not significantly low to be additive. The use of salt as a flavour enhancer does not in any way diminish the antimicrobial activity of the plant.

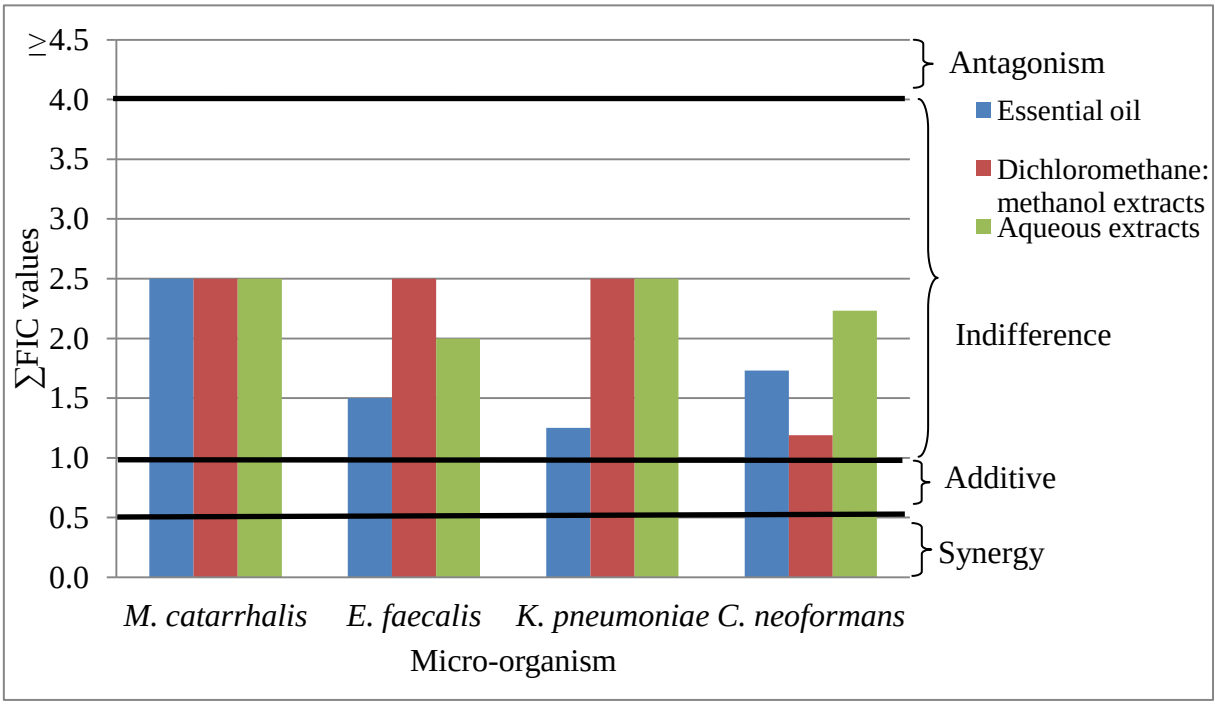


Figure 9.6 The interaction of *A. afra* with salt.

One can assume that the addition of these said adjuncts are possibly used for other purposes by traditional healers. Some of the possible uses include the use of honey to enhance taste, especially in children. Milk could be helpful in producing a possible enzymatic reaction. Salt could also be primarily added to mask unpleasant bitterness of the medicinal plant. Brandy and vinegar may be used in the making of poultices to create a cooling sensation when they evaporate or even just as a means to soften the leaf and roots of the plant.

9.2.5.1 The combination of *A. afra, Tetradenia riparia* and salt

Salt, together with *A. afra* and *T. riparia* is used as a decoction for the treatment of coughs (Hutchings et al., 1996). The use of salt in combination with plants is also mentioned in Adams et al. (2009) where it states that salt is used in combination with the crushed leaves of *Plantago lanceolata* and are then placed on arthritic limbs (Bock, 1577; Matthiolus, 1590; Lonicerus, 1770).

Furthermore, it is also worth considering that as the combination of *A. afra* with *T. riparia* and salt is typically ingested, the salt content may play a role in the dissolution and/or absorption of the active components. It is known that the salt form of drugs e.g. salicylic acid shows faster dissolution and absorption rates. Salts increase dissolution rates thus increasing absorption rates. When salt encounters aqueous environments, it rapidly dissolves and takes the compounds bound to it with it in solution. This enables easier absorption (Kerns and Di, 2008). When used as an application to the skin, the salt may have a cooling effect or the rough texture may be beneficial in helping to stimulate blood flow in these areas. Thus, the administration of the combination is essential to ultimately understanding the possible benefits of these medicinal plants.

The antimicrobial activity of the combination of *T. riparia* with *A. afra* and salt has not been tested previously and represents a new area for further in depth analysis into the implications of administration methods and additives to the overall activity and effectiveness of medicinal plants

9.2.5.1.1 Chromatographic techniques

9.2.5.1.1.1 Essential oil composition of *T. riparia*

Essential oil was distilled from *T. riparia*, which was collected from the Walter Sisulu Botanical gardens in Johannesburg. Forty-two compounds were identified in the essential oil, representing 91.2% of the total composition (Table 9.7). The major compounds in the essential oil comprised of γ-terpinene (4.1%), β-caryophyllene (32.4%), germacrene D (6.3%), bicyclogermacrene (4.8%), and α-cadinol (5.4%).

Table 9.7 Essential oil composition of *T. riparia.*

RRI*	Compound name	Area (%)
1016	α-Pinene	0.1
1019	α-Thujene	0.1
1104	β-Pinene	0.1
1117	Sabinene	0.3
1193	α-Terpinene	1.9
1193	Limonene	0.1
1203	β –Phellandrene	0.1
1242	**γ-Terpinene**	**4.1**
1270	*p*-Cymene	1.0
1281	Terpinolene	1.1
1701	Verbenene	0.1

RRI*	Compound name	Area (%)
1456	*trans* Sabinene hydrate	3.4
1448	α-Copaene	0.3
1511	β-Bourbonene	1.2
1528	α-Gurjunene	0.5
1546	Linalool	0.2
1562	Terpinen-4-ol acetate	1.0
1586	β-Elemene	0.2
1589	β-Copaene	0.1
1595	**β-Caryophyllene**	**32.4**
1627	*cis*-Menth-2-en-1-ol	0.7
1647	*allo* Aromadendrene	0.3
1674	α-Humulene	0.9
1701	α-Terpineol	0.7
1715	**Germacrene D**	**6.3**
1728	α-Muurolene	0.8
1741	**Bicyclogermacrene**	**4.8**
1763	δ-Cadinene	2.1
1768	γ-Cadinene	0.4
1861	*p*-Cymen-9-ol	0.2
1900	Cubebol	0.1
1948	Palustrol	2.6
2009	Caryophyllene oxide	0.7
2067	Germacrene-4-D-ol	0.2
2084	Cubenol	0.5
2103	Guaiol	0.1
2141	Spathulenol	0.5
2185	T-Cadinol	1.1
2196	Eusdesmol	2.4
2201	**α-Cadinol**	**5.4**
2327	**Undetermined***	**9.8**
	Abiatatriene (t)	2.3
Total		91.2

RRI*: Relative retention indices calculated against n-alkanes.
Area (%) calculated from FID data.
Tr = Trace; *UD: Undetermined. Major peaks: 288, 273, 245, t: tentative identification.

Campbell et al. (1997) have reported on the composition of the essential oil of *T. riparia* from South Africa. Thirty-five compounds were identified and the main constituents found in the study were α-terpineol (22.6%), fenchone (13.6%), β-fenchyl alcohol (10.7%), β-caryophyllene (7.9%) and perillyl alcohol (6.0%). A study by Omolo et al. (2004) revealed the major compound present in the essential oil of *T. riparia* from Kenya to be fenchone (±65%), limonene (±2.0%) and 1, 8-cineole (1.5%).

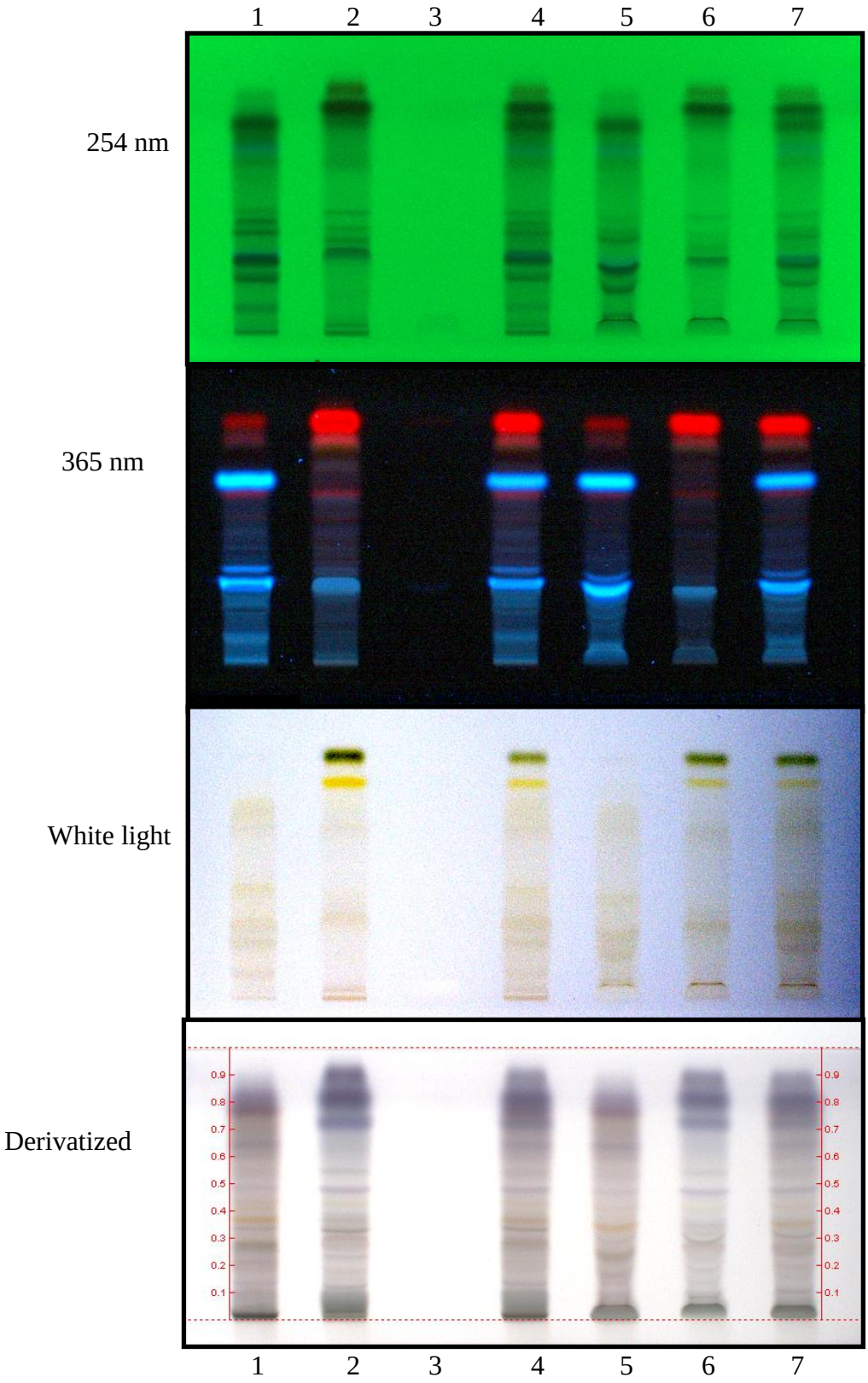
A recent study (Gazim et al., 2010) was carried out on the seasonal variation, chemical composition and analgesic and antimicrobial properties of the essential oil from the leaves of *T. riparia* in Brazil. The authors reported a total of 36 compounds identified from the essential oil, accounting for 95.0-99.6% of the volatile constituents. The major constituents found were 14-hydroxy-9-epi-caryophyllene (±20%), cis-muurolol-5-en-4-α-ol (±10%), α-cardinol (±6%), ledol (±6%), calyculone (±20%), abietadiene (±10%) and fenchone (±10%).

The current study showed the major compound present as β-caryophyllene at 32.4% with an unidentified compound at 9.8%. This is different from the composition found by Campbell et al. (1997), where β-caryophyllene was only found at 7.9% and Gazim et al. (2010) found β-caryophyllene in small amounts only. Omolo et al. (2004) did not find any β-caryophyllene in the essential oil at all. In fact, fenchone was the main compound in Omolo et al. (2004), but no trace was found in the essential oil of *T. riparia* in this study. The differences in the constituents of the essential oils of *T. riparia* in South Africa and Kenya, and Brazil may be due to differences in environmental factors, chemotype, nutrition of the plants and genetics (Gazim et al., 2010).

9.2.5.1.1.2 Thin layer chromatography

In previous TLC assays, two major bands were found for *T. riparia* (Scott et al., 2004). A grey band with an R_f value of 0.36 (in a toluene: diethyl ether: 1.75 M acetic acid solvent) and 0.76 and another which was the 1,8-cineole marker at R_f value of 0.79 which was light blue in colour (Scott et al., 2004; Scott and Springfield, 2004).

Similar bands were noted in the *T. riparia* extracts in the current study. The same three major bands were visible in Figure 9.7, at 254 nm, 365 nm and after derivatization. These bands were also visible in the combination with salt (Figure 9.7, lane 7) and in the triple combination.



1	*A. afra*
2	*T. riparia*
3	Salt
4	*A. afra* with *T. riparia*
5	*A. afra* with salt
6	*T. riparia* with salt
7	*A. afra*, *T. riparia* and salt

Figure 9.7 TLC plates of the dichloromethane: methanol extracts of *A. afra*, *Tetradenia riparia* with salt alone and in combination at 254 nm, 365 nm, white light and visualized with vanillin-sulphuric acid reagent.

9.2.5.1.2 Antimicrobial analysis

9.2.5.1.2.1 Essential oils

In Table 9.8, the MIC values of the essential oils of *T. riparia* alone and in combination with either salt or *A. afra* or all three together. For studies on *T. riparia* noteworthy activity was seen against *C. neoformans* (MIC value = 2.0 mg/ml), *S. agalactiae* (MIC value = 1.5 mg/ml) and *M. smegmatis* (MIC value = 2.0 mg/ml).

The combination of the essential oils of *A. afra* with *T. riparia* demonstrated noteworthy activity against *C. neoformans, S. agalactiae* and *M. smegmatis.* Salt showed no antimicrobial activity against *K. pneumoniae, M. catarrhalis, E. faecalis, C. neoformans, S. pneumoniae* and *S. pyogenes* at the highest concentration tested. However, some activity was noted against *S. agalactiae* and noteworthy activity was seen against *M. smegmatis* (MIC value = 0.6 mg/ml).

The combination of *A. afra* with salt showed noteworthy activity only against *M. smegmatis* (MIC value of 0.6 mg/ml) was obtained. For the combination of *T. riparia* with salt, some antimicrobial activity was noted. Noteworthy activity was seen against *M. smegmatis* with a MIC value of 1.2 mg/ml.

For the combination of *A. afra, T. riparia* and salt noteworthy antimicrobial activity was noted against *C. neoformans, S. pneumoniae* and *M. smegmatis*. The most significant activity was noted for M. *smegmatis* (MIC value of 0.2 mg/ml). ∑FIC values calculated revealed and synergy against *M. smegmatis*. Tentative interpretations showed varied interactions ranging from synergism to antagonism.

9.2.5.1.2.2 Dichloromethane: methanol extracts

In table 9.9 the results of the dichloromethane: methanol extracts of *A. afra* and *T. riparia* alone and in combination with salt are presented. For *T. riparia* independently noteworthy inhibitory activity were noted against *C. neoformans, S. pneumoniae* and *S. pyogenes* and *S. agalactiae* (MIC values of 0.5, 0.3, 0.4 and 0.6 mg/ml respectively).

Moderate inhibitory activity was noted for the combination of *A. afra* with *T. riparia* against *K. pneumoniae, M. catarrhalis, E. faecalis* and *M. smegmatis*. Noteworthy strong inhibition was obtained against *S. pneumoniae* with a MIC value of 0.5 mg/ml.

The combination of *A. afra* with salt showed some activity against *C. neoformans* and *S. agalactiae* and noteworthy moderate inhibition against *M. smegmatis* (MIC value = 0.7 mg/ml). For the combination of *T. riparia* with salt, noteworthy activity for the combination was obtained against *C. neoformans* with a MIC value of 0.3 mg/ml.

MIC values obtained for the triple combination of *A. afra* with *T. riparia* and salt showed noteworthy activity (values range between 0.1 and 0.5 mg/ml) against all the pathogens tested with the exception of *E. faecalis* and *S. agalactiae* where poor activity was noted. The ∑FIC values calculated showed antagonism against *S. agalactiae* and additivity against *M. smegmatis*. Tentative interpretations of the interactions of the triple combination showed synergistic activity against *K. pneumoniae* and *M. catarrhalis*.

9.2.5.1.2.3 Aqueous extracts

Table 9. 10 shows the MIC values obtained for the aqueous extracts of the combination of *A. afra* with *T. riparia* and salt against the eight pathogens tested. *T. riparia* aqueous extract showed moderate inhibition against *E. faecalis* (MIC value = 1.5 mg/ml).

The triple combination of the aqueous extracts showed some activity against *K. pneumoniae, M. catarrhalis, E. faecalis* and *C. neoformans*. Noteworthy strong inhibition was evident against *M. smegmatis* (MIC value of 0.5 mg/ml). ∑FIC values calculated showed synergy against *M. smegmatis* with a ∑FIC value of 0.3.

A previous antimicrobial study on *T. riparia* (Boily and van Puyvelde, 1986), an initial screening of activity, where the agar dilution method was used. The methanol extracts (80%) of the leaves, stems, root fruit, and flowers of *T. riparia* were tested against *Bacillus subtilis, Candida albicans, M. smegmatis, P. aeruginosa, S. aureus,* and *Salmonella gallinarum*. The leaves inhibited the growth of *B. subtilis, C. albicans, M. smegmatis,* and *P. aeruginosa*. The stems did not show any inhibitory activity. The roots inhibited only *B. subtilis* and *S. aureus,* while the fruits only inhibited the growth of *C. albicans*. The flowers showed good antimicrobial action by inhibiting *B. subtilis, C. albicans, M. smegmatis,* and *S. aureus*.

Table 9.8 MIC and ∑FIC values of the essential oils *A. afra*, *T. riparia*, and salt alone and in combination.

Micro-organism	MIC (mg/ml)/)∑FIC value indicated in brackets where applicable							∑FIC	
	A. afra	***T. riparia***	***A. afra* with *T. riparia***	**Salt (2%)**	***A. afra* with salt**	***T. riparia* with salt**	***A. afra, T. riparia* with salt**	***A. afra, T. riparia* with salt**	**Interpretation**
K. pneumoniae NCTC 9633	8.0	7.0	8.0 (1.1)	≥5.0	9.3 (ND[1])	9.3 (ND[1])	12.3	ND[1]	Indifference/Antagonism[2]
M. catarrhalis ATCC 23246	8.7	8.0	8.0 (1.0)	≥5.0	9.3 (ND[1])	9.3 (ND[1])	12.3	ND[1]	Indifference/Antagonism[2]
E. faecalis ATCC 29212	8.7	32.0	8.0 (0.6)	≥5.0	18.5 (ND[1])	3.8 (ND[1])	12.3	ND[1]	Indifferent[2]
C. neoformans ATCC 90112	3.8	**2.0**	**1.0 (0.4)**	≥5.0	2.3 (ND[1])	2.3 (ND[1])	**1.4**	ND[1]	Indifferent[2]
S. pneumoniae ATCC 49619	3.3	8.0	4.0 (0.9)	≥5.0	18.5 (ND[1])	9.3 (ND[1])	**1.4**	ND[1]	Synergistic/Additive[2]
S. pyogenes ATCC 8668	**1.7**	6.0	6.0 (2.3)	≥5.0	9.3 (ND[1])	9.3 (ND[1])	4.3	ND[1]	Indifferent[2]
S. agalactiae ATCC 55618	4.8	**1.5**	**2.0** (0.9)	3.8	9.3 (2.2)	2.3 (1.1)	5.8	2.2	Indifferent
M. smegmatis (clinical)	1.5	**2.0**	**1.0** (0.6)	**0.6**	**0.6** (0.7)	**1.2** (1.2)	**0.2**	**0.2**	**Synergy**

*Values given in bold indicate noteworthy activity.
ND[1] No ∑FIC value could be calculated as no MIC end point for salt/combination was obtained at the highest concentration tested.
[2] Tentative interpretation according to MIC data.
Shaded area: results previously discussed in Chapter 3 but included here for reference purposes.
Controls excluded for the sake of brevity. Please refer to Chapter 3 for further detail regarding control values.

Table 9.9 MIC and ∑FIC values of the dichloromethane: methanol extracts of *A. afra*, *T. riparia*, and salt alone and in combination.

Micro-organism	MIC (mg/ml))/∑FIC value indicated in brackets where applicable							∑FIC	
	A. afra	***T. riparia***	***A. afra* with *T. riparia***	**Salt (2%)**	***A. afra* with salt**	***T. riparia* with salt**	***A. afra, T. riparia* and salt**	***A. afra, T. riparia* and salt**	**Interpretation**
K. pneumoniae NCTC 9633	6.3	1.3	1.0 (0.5)	≥5.0	10.5 (ND[1])	≥10.5 (ND[1])	**0.1**	ND[1]	Synergy[2]
M. catarrhalis ATCC 23246	4.7	4.0	1.0 (0.2)	≥5.0	10.5 (ND[1])	≥10.5 (ND[1])	**0.1**	ND[1]	Synergy[2]
E. faecalis ATCC 29212	6.3	1.3	1.5 (0.7)	≥5.0	≥10.5 (ND[1])	≥10.5 (ND[1])	6.2	ND[1]	Indifference/Antagonism[2]
C. neoformans ATCC 90112	1.2	**0.5**	2.0 (2.8)	≥5.0	2.6 (ND[1])	**0.3** (ND[1])	**0.4**	ND[1]	Indifference/Additive[2]
S. pneumoniae ATCC 49619	**0.5**	**0.3**	**0.5** (1.3)	≥5.0	≥10.5 (ND[1])	≥10.5 (ND[1])	**0.4**	ND[1]	Indifferent[2]
S. pyogenes ATCC 8668	1.1	**0.4**	8.0 (3.6)	≥5.0	≥10.5 (ND[1])	≥10.5 (ND[1])	**0.8**	ND[1]	Indifference/Additive[2]
S. agalactiae ATCC 55618	3.0	**0.6**	4.0 (4.0)	3.8	4.9 (1.5)	2.6 (2.5)	6.2	4.7	Antagonistic
M. smegmatis (clinical)	1.7	1.0	1.0 (0.8)	**0.6**	**0.7** (0.7)	1.3 (1.7)	**0.5**	0.6	Additive

*Values given in bold indicate noteworthy activity.
ND[1] No ∑FIC value could be calculated as no MIC end point for salt/combination was obtained at the highest concentration tested.
[2] Tentative interpretation according to MIC data.
Shaded area: results previously discussed in Chapter 3 but included here for reference purposes.
Controls excluded for the sake of brevity. Please refer to Chapter 3 for further detail regarding control values.

Table 9.10 MIC and ∑FIC values of the aqueous extracts *A. afra, T. riparia,* and salt alone and in combination.

Micro-organism	MIC (mg/ml))/∑FIC value indicated in brackets where applicable							∑FIC	
	A. afra	***T. riparia***	***A. afra* with *T. riparia***	**Salt (2%)**	***A. afra* with salt**	***T. riparia* with salt**	***A. afra, T. riparia* and salt**	***A. afra, T. riparia* and salt**	**Interpretation**
K. pneumoniae NCTC 9633	12.0	≥16.0	2.0 (ND[1])	≥5.0	5.3 (ND[1])	2.6 (ND[1])	3.1	ND[1]	Synergy[2]
M. catarrhalis ATCC 23246	8.0	2.5	≥16.0 (ND[1])	≥5.0	10.5 (ND[1])	5.3 (ND[1])	6.2	ND[1]	Indifferent[2]
E. faecalis ATCC 29212	7.0	1.5	≥16.0 (ND[1])	≥5.0	10.5 (ND[1])	2.6 (ND[1])	6.2	ND[1]	Indifferent[2]
C. neoformans ATCC 90112	4.0	2.0	4.0 (1.5)	≥5.0	2.6 (ND[1])	2.6 (ND[1])	1.6	ND[1]	Indifference/Additive[2]
S. pneumoniae ATCC 49619	8.0	≥16.0	≥16.0 (ND[1])	≥5.0	≥10.5 (ND[1])	≥10.5 (ND[1])	≥10.5	ND[1]	Indifference/Antagonism[2]
S. pyogenes ATCC 8668	5.3	≥16.0	8.0 (ND[1])	≥5.0	≥10.5 (ND[1])	10.5 (ND[1])	≥10.5	ND[1]	Indifference/Antagonism[2]
S. agalactiae ATCC 55618	1.7	≥16.0	≥16.0 (ND[1])	3.8	≥10.5 (ND[1])	≥10.5 (ND[1])	≥10.5	ND[1]	Indifferent[2]
M. smegmatis (clinical)	8.0	6.0	2.0 (0.3)	1.3	2.6 (2.4)	9.8 (8.9)	**0.5**	**0.3**	**Synergy**

*Values given in bold indicate noteworthy activity.
ND[1] No ∑FIC value could be calculated as no MIC end point for salt/combination was obtained at the highest concentration tested.
[2] Tentative interpretation according to MIC data.
Shaded area: results previously discussed in Chapter 3 but included here for reference purposes.
Controls excluded for the sake of brevity. Please refer to Chapter 3 for further detail regarding control values.

In 1995, Vlietinck et al. tested the antimicrobial activity of the methanol extracts of *T. riparia* where the best activity was obtained against a yeast. This is in partial congruence with the current study where good results were obtained against the yeast, *C. neoformans*. Later, McGaw et al. (2000) tested the antimicrobial activity of the methanol, hexane, and aqueous extracts, of *T. riparia* using the disc diffusion method. No activity, against the pathogens was noted using the discs thus further microplate MIC determination was not carried out. Comparisons with the current study are difficult as the method of antimicrobial testing differs.

A recent study (Gazim et al., 2010) tested the antimicrobial activity of the essential oil of *T. riparia* using the disc diffusion method followed by the measurement of the MIC values using the microdilution MIC method. The most sensitive micro-organisms were *S. aureus, B. subtilis,* and *C. albicans* with the largest inhibition zones and the lowest MIC values. These antimicrobial activities obtained of *T. riparia* were highest in the Summer, then Autumn, Winter and the lowest in Spring. MIC values obtained against *S. aureus* ranged from 15.6-31.2 µg/ml, against *B. subtilis* MIC values obtained were between 7.8-15.6 µg/ml and against *C. albicans* MIC values were from 31.2-62.4 µg/ml. Good activity was also seen against other organisms tested i.e. *E. faecalis, P. aeruginosa, E. coli, K. pneumoniae* and *S. enterica* with MIC values of 62.5, 125.0 and 250 µg/ml respectively. In the current study the most significant activity was noted for the essential oil of *T. riparia* against *S. agalactiae* with a MIC value of 1.5 mg/ml.

The active principles of *T. riparia* which display significant antimicrobial properties are the 8(14), 15-sandaracopimaradiene-7α, 18-diol (De Kimpe et al., 1982; van Puyvelde, 1983; Boily and van Puyvelde, 1986; van Puyvelde et al., 1986).

Variation between the results previously obtained could be due to the different localities of the *T. riparia* collected. Boily and van Puyvelde (1986) and Vleitinck et al. (1995) tested *T. riparia* collected from Rwanda and Gazim et al. (2010) tested *T. riparia* oil from Brazil. McGaw et al. (2000) tested the extracts of *T. riparia,* which was collected from South Africa. The *T. riparia* collected in this study was also from South Africa and the results obtained were moderate to poor. Thus this correlates with the findings of the antimicrobial activity that was obtained for the South African collections

9.3 Conclusions

- The combination of the dichloromethane; methanol extracts *A. afra* with orange blossom honey (100%), wild blossom honey (100%) and blue gum honey (100%) all showed synergy against *C. neoformans*.
- The essential oils in combination with orange blossom, wild blossom and blue gum honey are antagonistic against all the pathogens tested with the exception of the combination of *A. afra* with wild blossom honey (100%) and blue gum honey (100%) against *C. neoformans.*
- Additivity is seen for *A. afra* essential oil with milk (skim and or full cream) against *C. neoformans*, *M. catarrhalis* and *K. pneumoniae*.
- The dichloromethane: methanol extracts of *A. afra* are additive in combination with vinegar against *E. faecalis* and *K. pneumoniae*.
- The dichloromethane; methanol extracts of *A. afra* with brandy show additivity against *M. catarrhalis* and *C. neoformans*. The essential oils and aqueous extracts in combination with brandy were antagonistic against *M. catarrhalis*, *E. faecalis* and *K. pneumoniae*.
- Salt in combination with *A. afra* displays no significant interaction. Salt in the combination of *A. afra* with *T. riparia* could therefore be incorporated into the preparation to augment the taste of these two unpalatable plants in one remedy.
- The addition of an adjunct to *A. afra* by traditional healers is most likely due to other factors like enhancing taste or preventing bitterness.
- The results obtained from the triple combination of the essential oils i.e. *A. afra, T. riparia,* and salt, showed the noteworthy and very strong inhibition against *M. smegmatis* with a MIC value of 0.2 mg/ml. ∑FIC values obtained showed synergy against *M. smegmatis* and antagonism against *S. agalactiae.*
- The dichloromethane: methanol extracts of the combination of *A. afra, Tetradenia riparia,* and salt showed good activity with noteworthy inhibition against all the pathogens tested except against *E. faecalis* and *S. agalactiae.* The best activity as noted against *K. pneumoniae* and *M. catarrhalis* with MIC values of 0.1 mg/ml. ∑FIC values showed antagonism against *S. agalactiae.*
- The aqueous extracts of the combination of *A. afra, Tetradenia riparia* and salt showed noteworthy activity against *M. smegmatis* with a MIC value of 0.5 mg/ml.

Chapter 10

Summary and general conclusions

Traditional healers often use combinations of plants or adjuncts with *A. afra* to treat common respiratory complaints. This study was conducted with the aim of validating the traditional use of these combinations from an antimicrobial perspective, in order to provide valuable insight into the rationale for the use of these herbal combinations.

This research has provided a direction for further studies into the more complex uses of plant combinations, particularly with respect to the principle plant *A. afra.* An in-depth literature review was conducted and combinations of plants commonly used by traditional healers to treat respiratory infections were identified (Table 1.1). These were elaborated on and explored in Chapter 1. Five double combinations and four triple combinations were selected for further investigation.

All aromatic plants where essential oil could be obtained were analysed by GC-MS and the detailed compositional analysis is provided with the corresponding plants in the thesis and monographs. A summary of the main compounds present the six plants essential oils evaluated is given in Table 10.1.

Table 10.1 Summary of the major chemical constituents found from all plant essential oils in this study.

Essential oil	Major compounds	% of major compound
A. betulina	isomenthone	31.4
	limonene	18.9
	menthone	10.0
	diosphenol	8.1
	pseudodiosphenol	7.5
A. afra	camphor	41.0
	1,8-cineole	17.0
	borneol	8.6
	β-thujone	6.0
	artemisia ketone	6.4
	camphene	4.0

Essential oil	Major compounds	% of major compound
E. globulus	1,8-cineole	63.0
	α-pinene	16.7
	limonene	3.6
	ρ-cymene	2.7
L. javanica	linalool	70.7
	caryophyllene oxide	6.9
	trans-linalool oxide	4.5
	cis-linalool oxide	4.0
	ρ-cymene	3.4
	1,8-cineole	2.3
O. asteriscoides	1,8-cineole	59.0
	camphor	8.2
	α-terpineol	7.2
T. riparia	β-caryophyllene	32.4
	germacrene D	6.3
	α-cardinol	5.4
	bicyclogermacrene	4.8
	γ-terpinene	4.1

The TLC analysis of the plant essential oils, dichloromethane: methanol and aqueous extracts showed good separation of the compounds and the complexity of the plants individually and in combination were illustrated.

MIC values for the essential oils and extracts that produced the highest activity are shown in Table 10.2. The most significant activity noted for plants studied independently was that from *E. globulus* dichloromethane: methanol extracts against *M. catarrhalis* with a MIC value of 0.01 mg/ml.

From the antimicrobial interactions observed in Table 10.3, the most synergistic combinations in a 1:1 ratio is observed when *A. afra* is combined with *O. asteriscoides* (dichloromethane: methanol extracts) against *S. pyogenes*, when *A. afra* is combined with *E. globulus* (aqueous extracts) against *S. pneumoniae*, when *L. randii* is combined with *E. globulus* (dichloromethane: methanol extracts) against *K. pneumoniae* and when the aqueous extracts are combined against *M. smegmatis* in the combination of *A. afra* with *T. riparia* (dichloromethane: methanol extracts) against *M. catarrhalis*, all with an ∑FIC value of 0.2.

Table 10.2 Summary of the most significant antimicrobial activity of the plant samples tested independently.

Plant	Plant preparation	Micro-organism	MIC value (mg/ml)
A. afra	Essential oil	*M. smegmatis* (clinical)	1.5
	Dichloromethane: methanol extract	*S. pneumoniae* (ATCC 49619)	0.5
L. javanica	Essential oil	*S. pyogenes* (ATCC 8668)	1.4
	Dichloromethane: methanol extract	*S. pneumoniae* (ATCC 49619)	0.4
		S. pyogenes (ATCC 8668)	
	Aqueous extract	*S. agalactiae* (ATCC 55618)	0.5
O. asteriscoides	Essential oil	*S. pneumoniae* (ATCC 49619)	1.0
	Dichloromethane: methanol extract	*C. neoformans* (ATCC 90112)	0.3
	Aqueous extract	*C. neoformans* (ATCC 90112)	0.5
A. betulina	Essential oil	*S. pyogenes* (ATCC 8668)	1.0
E. globulus	Essential oil	*C. neoformans* (ATCC 90112)	0.6
	Dichloromethane: methanol extract	*M. catarrhalis* (ATCC 23246)	0.01
	Aqueous extract	*C. neoformans* (ATCC 90112)	0.8
L. randii	Dichloromethane: methanol extract	*M. smegmatis* (clinical)	0.5
Z. capense	Dichloromethane: methanol extract	*C. neoformans* (ATCC 90112)	0.4
	Aqueous extract	*S. agalactiae* (ATCC 55618)	0.4
T. riparia	Essential oil	*S. agalactiae* (ATCC 55618)	1.5
	Dichloromethane: methanol extract	*S. pneumoniae* (ATCC 49619)	0.3

The triple combination of *A. afra* with *T. riparia* (essential oil) with salt was also synergistic (∑FIC value = 0.2) against *M. smegmatis*. The combination of *O. asteriscoides* and *E.*

globulus (essential oils) against *E. faecalis* and the combination of *A. afra* with *L. randii* (dichloromethane: methanol extracts) against *C. neoformans* showed synergy with ∑FIC values of 0.1.

Various ratios within the isobologram interpretations demonstrated synergy and the most significant was that for the combination of *A. afra* with *E. globulus* (aqueous extract) against *C. neoformans* wherein all ratios tested were synergistic.

Some antagonism was found and this could be important as these ratios could be highlighted as inadvisable for traditional healing practises. The interaction that was most antagonistic in a 1:1 ratio of the combination of *O. asteriscoides* with *E. globulus* (dichloromethane: methanol extracts) against *M. catarrhalis* where a ∑FIC value of 77.3 was obtained. The isobologram studies demonstrated the highest incidence of antagonism was the combination of *A. afra* with *E. globulus* (dichloromethane: methanol extracts) against *M. catarrhalis* wherein five of the nine ratios were antagonistic.

Table 10.3 Summary of the most significant interactions present in the plant combinations tested.

Plant combination	**Plant part/product**	**Micro-organism**	**∑FIC value/isobologram ratios**	**Interaction**
A. afra with *L. javanica*	Dichloromethane: methanol extracts	*S. agalactiae* (ATCC 55618)	∑FIC value = 0.4	Synergy
		M. catarrhalis (ATCC 23246)	Isobologram (ratios 2:8 and 1:9)	Synergy
		E. faecalis (ATCC 29212)	Isobologram (ratio 1:9)	Synergy
A. afra with *O. asteriscoides*	Dichloromethane: methanol extracts	*S. pneumoniae* (ATCC 49619)	∑FIC value = 0.3	Synergy
		S. pyogenes (ATCC 8668)	∑FIC value = 0.2	Synergy
		M. smegmatis (clinical)	∑FIC value = 0.3	Synergy
	Aqueous extract	*M. catarrhalis* (ATCC 23246)	Isobologram (ratios 4:6, 3:7, 2:8 and 1:9)	Synergy
		K. pneumoniae (NCTC 9633)	Isobologram (ratio 8:2)	Synergy

Plant combination	Plant part/product	Micro-organism	∑FIC value/isobologram ratios	Interaction
		E. faecalis (ATCC 29212)	Isobologram (ratio 9:1)	Synergy
		C. neoformans (ATCC 90112)	Isobologram (ratios 9:1, 8:2, 6:4, 4:6, 3:7, 2:8 and 1:9)	Synergy
	Essential oil	*C. neoformans* (ATCC 90112)	Isobologram (ratio 8:2)	Synergy
A. afra with *A. betulina*	Dichloromethane: methanol extracts	*M. catarrhalis* (ATCC 23246)	∑FIC value = 0.3	Synergy
			Isobologram (ratios 3:7, 4:6 and 1:1)	Synergy
A. afra with *E. globulus*	Dichloromethane: methanol extracts	*K. pneumoniae* (NCTC 9633)	∑FIC value = 0.5	Synergy
		S. pyogenes (ATCC 8668)	∑FIC value = 0.3	Synergy
		M. smegmatis (clinical)		Synergy
		M. catarrhalis (ATCC 23246)	∑FIC value = 5.8	Antagonism
		E. faecalis (ATCC 29212)	∑FIC value = 7.6	Antagonism
	Aqueous extract	*C. neoformans* (ATCC 90112)	∑FIC value = 0.3	Synergy
		S. pneumoniae (ATCC 49619)	FIC value = 0.2	Synergy
	Dichloromethane: methanol extracts	*M. catarrhalis* (ATCC 23246)	Isobologram (ratios 8:2, 7:3, 6:4, 1:1 and 4:6)	Antagonism
		K. pneumoniae (NCTC 9633)	Isobologram (ratios 4:6 and 1:1)	Synergy
		E. faecalis (ATCC 29212)	Isobologram (ratios 6:4 and 1:1)	Antagonism
	Aqueous extract	*M. catarrhalis* (ATCC 23246)	Isobologram (all ratios except 1:1)	Synergy
		C. neoformans (ATCC 90112)	Isobologram (all ratios)	Synergy
O. asteriscoides with *E. globulus*	Essential oil	*E. faecalis* (ATCC 29212)	∑FIC value = 0.1	Synergy
	Dichloromethane: methanol extracts	*M. catarrhalis* (ATCC 23246)	∑FIC value = 77.3	Antagonism
		E. faecalis (ATCC 29212)	∑FIC value = 34.0	Antagonism

Plant combination	Plant part/product	Micro-organism	∑FIC value/isobologram ratios	Interaction
		S. agalactiae (ATCC 55618)	∑FIC value = 0.4	Synergy
A. afra, *O. asteriscoides* with *E. globulus*	Essential oil	*C. neoformans* (ATCC 90112)	∑FIC value = 6.5	Antagonism
	Dichloromethane: methanol extracts	*K. pneumoniae* (NCTC 9633)	∑FIC value = 0.5	Synergy
		S. agalactiae (ATCC 55618)	∑FIC value = 0.4	Synergy
		M. catarrhalis (ATCC 23246)	∑FIC value = 57.6	Antagonism
		E. faecalis (ATCC 29212)	∑FIC value =42.9	Antagonism
A. afra with *L. randii*	Dichloromethane: methanol extracts	*C. neoformans* (ATCC 90112)	∑FIC value = 0.1	Synergy
		S. pyogenes (ATCC 8668)	∑FIC value = 6.0	Antagonism
L. randii with *E. globulus*	Dichloromethane: methanol extracts	*K. pneumoniae* (NCTC 9633)	∑FIC value = 0.2	Synergy
		M. catarrhalis (ATCC 23246)	∑FIC value =36.6	Antagonism
		E. faecalis (ATCC 29212)	∑FIC value = 25.1	Antagonism
	Aqueous extract	*M. smegmatis* (clinical)	∑FIC value = 0.2	Synergy
A. afra, L. randii with *E. globulus*	Dichloromethane: methanol extracts	*K. pneumoniae* (NCTC 9633)	∑FIC value = 0.3	Synergy
		C. neoformans (ATCC 90112)	∑FIC value = 0.3	Synergy
		M. smegmatis (clinical)	∑FIC value = 0.5	Synergy
A. afra with *Z. capense*	Dichloromethane: methanol extracts	*S. pneumoniae* (ATCC 49619)	∑FIC value = 0.4	Synergy
		E. faecalis (ATCC 29212)	Isobologram (ratio 6:4)	Synergy
		C. neoformans (ATCC 90112)	Isobologram (ratio 7:3)	Synergy
A. afra with *T. riparia*	Essential oil	*C. neoformans* (ATCC 90112)	∑FIC value = 0.4	Synergy
	Dichloromethane: methanol extracts	*K. pneumoniae* (NCTC 9633)	∑FIC value = 0.5	Synergy

Plant combination	Plant part/product	Micro-organism	∑FIC value/isobologram ratios	Interaction
		M. catarrhalis (ATCC 23246)	∑FIC value = 0.2	Synergy
		S. pyogenes (ATCC 8668)	∑FIC value = 13.6	Antagonism
		S. agalactiae (ATCC 55618)	∑FIC value = 4.0	Antagonism
	Aqueous extract	*M. smegmatis* (clinical)	∑FIC value = 0.3	Synergy
T. riparia with salt	Aqueous extract	*M. smegmatis* (clinical)	∑FIC value = 8.9	Antagonism
A. afra, *T. riparia* with salt	Essential oil	*M. smegmatis* (clinical)	∑FIC value = 0.2	Synergy
	Dichloromethane: methanol extracts	*S. agalactiae* (ATCC 55618)	∑FIC value = 4.7	Antagonism
	Aqueous extract	*M. smegmatis* (clinical)	∑FIC value = 0.3	Synergy

Table 10.4 demonstrates a summary of the main results attained when *A. afra* was combined with adjuncts such as honey, milk, vinegar and brandy. The best combinational activity from the adjuncts tested in this study was obtained from the combination of *A. afra* (dichloromethane: methanol extracts) with orange blossom honey (100%), with wild blossom honey (100%) and blue gum honey (100%) against *C. neoformans*. All three interactions were synergistic. The highest antagonism noted were in the combinations of *A. afra* with orange blossom honey (100%), *A. afra* with vinegar and *A. afra* with brandy against *M. catarrhalis*, *E. faecalis*, *K. pneumoniae* and *C. neoformans*. Thus, care should be taken when combining these adjuncts with *A. afra*.

Table 10.4 Summary of the adjunct interactions with *A. afra*.

Adjunct in combination with *A. afra*	Plant part/product	Micro-organism	Interaction *
A. afra with orange blossom honey (64.0 mg/ml)	Aqueous extract	*C. neoformans* (ATCC 90112)	Additive
A. afra with orange blossom honey (100%)	Essential oil	*M. catarrhalis* (ATCC 23246)	Antagonism
		E. faecalis (ATCC 29212)	Antagonism

Adjunct in combination with *A. afra*	**Plant part/product**	**Micro-organism**	**Interaction ***
		K. pneumoniae (NCTC 9633)	Antagonism
		C. neoformans (ATCC 90112)	Antagonism
	Dichloromethane: methanol extracts	*C. neoformans* (ATCC 90112)	Synergy
	Aqueous extract	*E. faecalis* (ATCC 29212)	Antagonism
		C. neoformans (ATCC 90112)	Antagonism
		K. pneumoniae (NCTC 9633)	Additive
A. afra with wild blossom honey (100%)	Essential oil	*M. catarrhalis* (ATCC 23246)	Antagonism
		E. faecalis (ATCC 29212)	Antagonism
		K. pneumoniae (NCTC 9633)	Antagonism
	Dichloromethane: methanol extracts	*C. neoformans* (ATCC 90112)	Synergy
	Aqueous extract	*E. faecalis* (ATCC 29212)	Antagonism
		C. neoformans (ATCC 90112)	Antagonism
		K. pneumoniae (NCTC 9633)	Additive
A. afra with blue gum honey (100%)	Essential oil	*M. catarrhalis* (ATCC 23246)	Antagonism
		E. faecalis (ATCC 29212)	Antagonism
		K. pneumoniae (NCTC 9633)	Antagonism
	Dichloromethane: methanol extracts	*K. pneumoniae* (NCTC 9633)	Additive
		C. neoformans (ATCC 90112)	Synergy
	Aqueous extract	*E. faecalis* (ATCC 29212)	Antagonism
		C. neoformans (ATCC 90112)	Antagonism

Adjunct in combination with *A. afra*	**Plant part/product**	**Micro-organism**	**Interaction ***
A. afra with skim milk	Essential oil	*E. faecalis* (ATCC 29212)	Antagonism
		C. neoformans (ATCC 90112)	Additive
	Aqueous extract	*M. catarrhalis* (ATCC 23246)	Additive
		K. pneumoniae (NCTC 9633)	Antagonism
A. afra with full cream milk	Essential oil	*E. faecalis* (ATCC 29212)	Antagonism
	Aqueous extract	*E. faecalis* (ATCC 29212)	Antagonism
		K. pneumoniae (NCTC 9633)	Additive
A. afra with vinegar	Essential oil	*M. catarrhalis* (ATCC 23246)	Antagonism
		E. faecalis (ATCC 29212)	Antagonism
		K. pneumoniae (NCTC 9633)	Antagonism
	Dichloromethane: methanol extracts	*E. faecalis* (ATCC 29212)	Additive
		K. pneumoniae (NCTC 9633)	Additive
	Aqueous extract	*M. catarrhalis* (ATCC 23246)	Antagonism
		E. faecalis (ATCC 29212)	Antagonism
		K. pneumoniae (NCTC 9633)	Antagonism
A. afra with brandy	Essential oil	*M. catarrhalis* (ATCC 23246)	Antagonism
		E. faecalis (ATCC 29212)	Antagonism
		K. pneumoniae (NCTC 9633)	Antagonism
	Dichloromethane: methanol extracts	*M. catarrhalis* (ATCC 23246)	Additive
		C. neoformans (ATCC 90112)	Additive

Adjunct in combination with *A. afra*	Plant part/product	Micro-organism	Interaction *
	Aqueous extract	*M. catarrhalis* (ATCC 23246)	Antagonism
		E. faecalis (ATCC 29212)	Antagonism
		K. pneumoniae (NCTC 9633)	Antagonism

*Interactions based on data from bar graphs in Chapter 9.

The purpose of this study was to validate the traditional use of *A. afra* combinations for the treatment of respiratory tract infections. This outcome has been achieved as many of the plant combinations have demonstrated that the essential oils have the most additivity in their combinations and thus the delivery form of inhalation may ensure that these infections are dealt with at the site of the microbial colonisation. Further *in vivo* studies are needed to confirm this. The results obtained from the essential oils mostly support the common use of these plants in combination as an inhalant to treat respiratory conditions. This use dates back to the origins of time when smoke or steam from a burning or boiling plant was inhaled (Mohagheghzadeh et al., 2006). Conventional medicine has also employed this method by inhaling drugs into the lungs using a nebulizer or metered dose inhalers for the treatment of respiratory illnesses.

The dichloromethane: methanol extracts also showed some outstanding activity. However, some antagonism was also noted. Care should be exercised when administering tinctures as antagonism is possible. The aqueous extracts, surprisingly, showed good activity in some combinations. This corresponds with the traditional use of these combinations in the form of decoctions and infusions.

The overall outcome of this study is that a valid and scientific basis for the use of these plants in combination has been established and this could prove to pave the way for future studies in the fight against bacterial respiratory infections. The study has produced positive and encouraging results that largely supports the traditional use of combinations and hopefully set the precedent for future research in this area.

Recommendations for future studies

Further testing of the use of adjuncts in combination with *A. afra* (Table 10.4) could prove helpful in the development of cost-effective and efficacious treatments for primary healthcare. These adjuncts may prove their value not only as pharmaceutical additives, but may prove to have a pharmacological benefit. Some of these benefits may be an enhanced activity at receptor sites to achieve an amplified result. They may also serve to improve the dissolution of the drug, thereby increasing the absorption of the active component. Studying these effects could be beneficial in the future.

Time-kill assays to determine the exact interactive effect of the combinations over time should be carried out. Toxicity studies should be undertaken to determine if toxicity is reduced or enhanced when plants are combined. The anti-oxidant, anti-inflammatory, decongestant, expectorant, antihistamine and antitussive properties should also be investigated, as plant combination selection may not only be associated with improved antimicrobial activity but also due to other pharmacological properties. There is the possibility that two plants are selected with the intention to address infection (one plant) and another related symptom for example inflammation. Thus by combining plants, two pharmacological effects are achieved within one preparation. Studies conducted should relate the synergistic interactions encountered to specific modes of action.

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http://www.google.co.za/search?hl=en&biw=1280&bih=683&q=Artemisia+afra&as_q=combination&btnG=Search%C2%A0within%C2%A0results
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http://www.camag.com/v/products/development/adc2.html
http://www.camag.com/v/products/assemblies/herbal_kits5.html
http://www.camag.com/v/products/derivatation/derivatization.html

Appendix A

Monographs of plant species investigated

A1Agathosma *betulina* (P.J. Berguis) Pillans

Common names of *Agathosma betulina* (P.J. Berguis) Pillans are: boegoe (Afrikaans); buchu (English, Khoi; French, Italy); round leaf buchu; short buchu; ibuchu (Xhosa); Bucco (Germany) (van Wyk and Wink, 2004; Moolla, 2006; van Wyk et al., 2009).

A1.1 Botanical description of *A. betulina*

Agathosma betulina (Rutaceae) is a round leafed perennial shrub, with woody branches, of up to 2 meters in height. The leaves are small, with a rounded apex, which curves backwards, and are rich in conspicuous oil glands along the margins and lower surfaces. Thus, they are highly aromatic with a rich gold colour oil. The oil has a strong, sweetish peppermint like odour (Watt and Breyer-Brandwijk, 1962; Moolla and Viljoen, 2008). Flowers (Figure A1.1) are born at the ends, throughout August, September and October (Sigmond, 1838). They are small and star shaped in white or purple (Moolla, 2006; van Wyk et al., 2009).



Figure A1.1 *A. betulina* flowers (A.M. Viljoen).

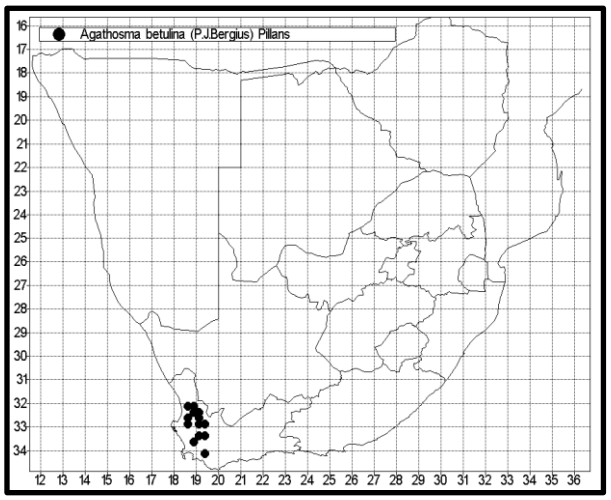


Figure A1.2 Botanical distribution of *A. betulina* (SANBI).

A1.2 Geographical locality of *A. betulina*

A. betulina is found in the sandy mountainous regions of the Cape of South Africa (Figure A1.2), specifically the Western Cape Province (Spreeth, 1976; Goldblatt and Manning, 2000; van Wyk and Gericke, 2000; Moolla, 2006; Moolla et al., 2007; van Wyk et al., 2009).

A1.3 Medicinal uses of *A. betulina*

A. betulina is said to be remedy for "almost every disease which afflicts man" (Watt and Breyer-Brandwijk, 1962). Buchu brandy (boegoebrandewyn) which comprises of buchu leaves infused in brandy is a preparation made by the natives of the Cape. This preparation is considered a supreme remedy for all chronic diseases as well as for acute diseases of the stomach and bladder (Sigmond, 1838; Watt and Breyer-Brandwijk, 1962; Simpson, 1998; Viljoen et al., 2006a). It is also used as an antibiotic for protection against infections (Forbes, 1986). The leaves are sometimes chewed, or mixed in brandy to make a tincture to relieve stomach complaints or as a stomachic tonic (Gentry, 1961; Watt and Breyer-Brandwijk, 1962; van Wyk and Wink, 2004; van Wyk et al., 2009). Buchu brandy is widely known and used as a diuretic and for the treatment of kidney and urinary tract diseases (Watt and Breyer-Brandwijk, 1962; Gentry, 1961; Moolla, 2006; van Wyk et al., 2009). Buchu leaves soaked in vinegar (buchu vinegar or boegoe-asyn) is used to wash and clean wounds (Moolla, 2006; van Wyk et al., 2009). Buchu is also used to treat the symptoms of rheumatism as well as gout (Simpson, 1998; Moolla, 2006; Viljoen et al., 2006a; van Wyk et al., 2009; Vermaak et al., 2009). The essential oil of buchu is known to have therapeutic properties such as alleviating fever, coughs, colds and flu (Simpson, 1998; Viljoen et al., 2006a).

A1.4 Chemical constituents of *A. betulina*

The essential oil of buchu has been studied (Gildmeister and Treibs, 1959; Fluck et al., 1961; Guenther, 1964; Kaiser et al., 1975; Blommaert and Bartel, 1976; Posthumus et al., 1996). These studies have attributed to its flavour to 8-mercapto-p-mentha-3-one. Two monoterpene thiols were identified to be responsible for the odour (Fluck et al., 1961; Gentry, 1961; Watt and Breyer-Brandwijk, 1962; Klein and Rojahn, 1967; Lamparsky and Schudel, 1971; Kaiser et al., 1975; Simpson, 1998).

Kaiser et al. (1975) isolated and identified 120 compounds in the commercial essential oil of buchu. The major compounds found were buchu camphor/diosphenol (15-30%), menthone and isomenthone (50-60%), limonene (±17%), pulegone and isopulegone (±7%). Viljoen et

al. (2006a) performed a study on the chemical composition of the commercially (Afriplex, South Africa) available *A. betulina* essential oil. They found the major constituents of buchu oil to be limonene (23.7%), pulegone (8.4%), menthone (29.2%) and isomenthone (14.2%).

Non-volatile major compounds in buchu leaf extract were identified as hesperidin and diosman (El-Shafae and El-Domiaty, 2001).

A2 *Allium sativum* L.

Allium sativum is also commonly known as garlic; ail blanc (French), Knoblauch (German), aglio (Italian), ajo (Spanish) and Lasan (Indian) (Ahmad and Beg, 2001; van Wyk and Wink, 2004). Allium species are said to be among the first cultivated crops in the world and its use as a spice and a medicinal plant dates back to antiquity (Sato and Miyata, 2000; Benkeblia, 2004; Rivlin, 2006).

A2.1 Botanical description of *A. sativum*

Allium sativum L. (Alliaceae) is a perennial shrub. It has fleshy slightly greyish leaves with rounded flower heads as well as numerous small bulbs, which are grouped and surrounded by a white papery sheath (Figure A2.1) (van Wyk and Wink, 2004).



Figure A2.1 bulbules of *A. sativum.*

A2.2 Geographical locality of *A. sativum*

Garlic is reported to have come from the Middle East or central Asia (van Wyk and Wink, 2004). However, the exact origin of garlic is unknown and thus only the cultivated form is commonly used.

A2.3 Medicinal uses of *A. sativum*

It has been used as a medicinal plant for over 4000 years and is still employed in folk medicine all over the world for the treatment of a variety of diseases (Block, 1985; Ali et al., 2000; Corzo-Martínez et al., 2007). The oldest literature recorded, on the use of garlic as a medicine and a condiment is from the Sumerians is dated at 2600-2100 BC (Harris et al., 2001). Garlic, in its various therapeutic forms i.e. powder, extracts or essential oils have been used in traditional medicine for various ailments by the Chinese, Egyptians, Greek, Indians, Koreans, Romans and the Africans (Stoll and Seebeck, 1951; Lucas, 1966; Cha, 1978;

Agarwal, 1996; Ankri and Mirelman, 1999; Jaber and Al-Mossawi, 2007; Abubakar, 2009). In Africa, garlic is used to treat abdominal discomfort, diarrhoea, otitis media, and respiratory tract infections (Ankri and Mirelman, 1999; Jaber and Al-Mossawi, 2007; Abubakar, 2009). The main or primary medicinal use of garlic is as a supportive dietary treatment of hyperlipidaemia (van Wyk et al., 2009). It is also used in the treatment and prevention of the symptoms associated with the common cold (Amagase, 2006; van Wyk et al., 2009). Garlic possesses antibacterial, antifungal, antiviral, antiprotozoal, anti-oxidant, anticarcinogenic and antimutagenic, antithrombotic and anti-arthritic effects (Sato and Miyata, 1999; Ali et al., 2000; Harris et al., 2001; Corzo-Martínez et al., 2007; Jaber and Al-Mossawi, 2007). Additionally, it has and is being used for tuberculosis, heart problems, headaches, bites, intestinal worms, and tumours (Watt and Breyer-Brandwijk, 1962; Block, 1985; Ali et al., 2000; Corzo-Martínez et al., 2007).

A2.4 Chemical constituents of *A. sativum*

Garlic contains a high amount of non-volatile organo-sulpur compounds, the primary one present is S-alk(en)yl-L-cysteine sulphoxides, such as alliin and γ-glutamylcysteines. Alliin is the main compound present in intact garlic which is degraded to allicin which is most often the main compound isolated in processed garlic materials (Ahmad and Beg, 2001; van Wyk and Wink, 2004). The main biological functions of garlic are due to the presence of their high organo-sulphur compounds i.e. e alliin (Augusti & Mathew, 1974; Amagase, 2006; Corzo-Martínez et al., 2007).

A3 *Artemisia afra* Jacq. ex Willd.

The common names of *A. afra* include African wormwood (English); umhlonyane, umhlonyane omcane (Xhosa, Zulu); lengana (Sotho, Tswana); als, alsem, wildeals (Afrikaans); armoise d'Afrique (French) and Afrikanischer Wermut (German) (Hutchings et al., 1996; van Wyk and Wink, 2004; van Wyk et al., 2009).

A3.1 Botanical description of *A. afra*

A. afra (Asteraceae) is an erect multi-stemmed perennial shrub. It grows up to two metres in height and it is a highly aromatic plant. It is bitter tasting and has a yellow-green volatile oil (von Koenen, 2001). It has feathery leaves (Figure A3.1) which are finely divided and have a greyish-green colour (van Wyk et al., 2009). In winter, the branches die, but rapidly regenerate from the base, and it blossoms from January to June (Hilliard, 1977; Liu et al., 2009; van Wyk et al., 2009). The flowers, which are borne along the branch ends, are pale yellowish and inconspicuous (Figure A3.2) (Hilliard, 1977; van Wyk et al., 2009).



Figure A3.1 Aerial leaves of *A. afra* (S.F. van Vuuren) with single feathery leaf of *A. afra* (inset) (A.M. Viljoen).



Figure A3.2 Pale yellowish flowers of *A. afra* (A.M. Viljoen).

A3.2 Geographical locality of *A. afra*

A. afra is a very common species and is indigenous to the mountainous regions in southern Africa (Figure A3.3) i.e. South Africa, Namibia, and Zimbabwe (Graven et al., 1992). It grows in the northern provinces of Gauteng and Limpopo along the eastern parts of South Africa as well as Swaziland and Lesotho extending up to the Western Cape of South Africa (Hilliard, 1977; Liu et al., 2009; van Wyk et al., 2009). It has an extensive distribution

northwards into tropical east Africa going as far north as Ethiopia (Gurib-Fakim, 2007; van Wyk et al., 2009).

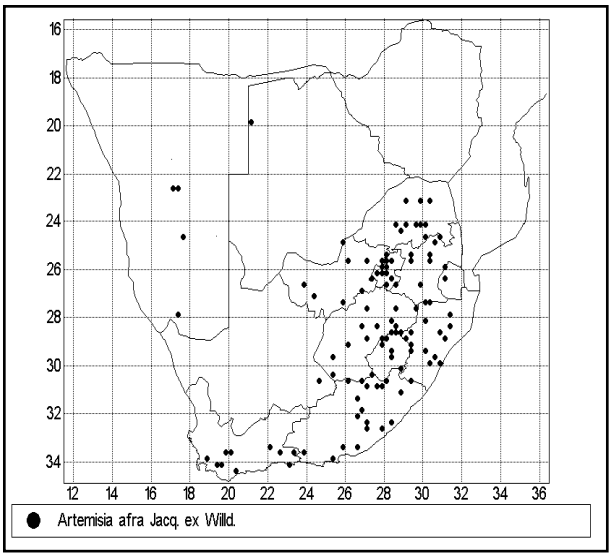


Figure A3.3 Geographical distribution of *A. afra* in South Africa (SANBI).

A3.3 Medicinal uses of *A. afr*a

A. afra is typically given as root or leaf infusions, which are drunk or used as a gargle. Leaf decoctions (with sugar or honey) and an alcohol extract are taken or the steam is inhaled. This is achieved by covering the container as well as the head with a towel (making a tent) thus blocking escape of the steam and then inhaling (Watt and Breyer-Brandwijk, 1962; Hutchings et al., 1996; von Koenen, 2001; Thring and Weitz, 2006; van Wyk et al., 2009). It has a wide spectrum of uses such as in the treatment of fever, chills, whooping cough, loss of appetite, headache, earache, skin ulcerations, intestinal worms, colic, indigestion, flatulence, constipation, gout, rheumatism, malaria and bladder and kidney disorders (Watt and Breyer-Brandwijk, 1962; von Koenen, 2001; van Wyk and Wink, 2004; Thring and Weitz, 2006; van Wyk et al., 2009). It is also employed to bring out the rash in measles (von Koenen, 2001).

A. afra is used regularly to treat respiratory problems, specifically both upper and lower respiratory tract infections (von Koenen, 2001). Among them are coughs, colds, inflammation of the throat, bronchitis, blocked nose, pneumonia and influenza (Watt and Breyer-Brandwijk, 1962; Hutchings et al., 1996; van Wyk et al., 2009).

A3.4 Chemical constituents of *A. afra*

Volatile as well as non-volatile constituents are found in *A. afra*. Non-volatile constituents from a pentane extract have revealed cerylcerotinate, nonacosane, friedeline and α- and β-amyrin (Silbernsgel et al., 1990; Graven et al., 1992) as well as surface flavonoids (methyl ethers of luteolin) (Wollenweber et al., 1989; van Wyk, 2008). Ascaridole, sesquiterpene lactone guaianolides, scopolectin and isofraxidin have also been identified (Jakupovic et al., 1988; Chagonda et al., 1997; Chagonda et al., 1999).

Graven et al. (1992) confirms the results on the main components of the volatile oil of *A. afra* from Zimbabwe by Priprzek et al. (1982) and Graven et al (1986) i.e. α-thujone (52.5%), β-thujone (13.1%), 1,8-cineole (13.0%) and camphor (6.6%). The study on the oil from Ethiopia by Worku and Rubiolo (1996) revealed the major compound to be artemisyl acetate (24.4-32.1%). In the Kenyan oil, 1,8-cineole was the major component at 63.37% (Mwangi et al., 1995) and α-thujone (52.5-54.2%) was the main component in the South African oil as analyzed by Lawrence (1989) and Libbey and Sturtz (1989). In the study by Chagonda et al. (1999), which studied the essential oil composition of cultivated *A. afra*, two chemotypes were identified i.e. cultivated from Harare and Murehwa. In the former chemotype, artemisia ketone (32.1-34.8%), camphor (21.8-24.4%), and 1,8-cineole (10.9-16.9%) were the dominant compounds. The latter sample contained 1,8-cineole (23.5-28.7%), camphor (20.2-21.3%) and borneol (14.2-17.0%) as the major compounds. The results of this study differed completely with the studies by Graven et al. (1992), Lawrence (1989) and Libbey and Sturtz (1989) which reported α-thujone as a major constituent of the essential oil of *A. afra*. These results are congruent with the studies by Moody et al. (1994) and Garnero (1977) (Chagonda et al., 1999). Mangena and Muyima (1999) studied the essential oil of *A. afra* from Fort Hare in South Africa and found major constituents to be α-thujone (78.68%), β-thujone (13.3%) and 1,8-cineole (8.19%). Once again, results differed from that of Graven et al., (1992).

In 2001, Burits et al. analyzed the essential oil of *A. afra* from Addis Ababa, Ethiopia using GC-MS. The major compounds found were camphor (26.8%), bornyl acetate (3.8%), 4-terpineol (3.6%) and chamazulene (3.2%). Muyima et al. (2002) found camphor (15.6%), 1, 8-cineole (13.6%) and β-thujanone (52.6%) to be the major compounds in the oil isolated from Fort Hare in South Africa. Viljoen et al. (2006b) studied the geographical variation by, GC-MS, of *A. afra* essential oil by comparing four natural plant populations i.e. Setibang (Lesotho), Giant's Castle (KwaZulu Natal), QwaQwa (Free State) and Kliprviersberg

(Gauteng). Different plants within a population were also collected. Differences in major compounds were noted e.g. 1,8-cineole which was present in high concentrations (50%) in the Gauteng samples was low (2%) in the Lesotho population. Inter-population differences also occurred wherein α-thujone was a major compound (77.7%) in plant sample C from Setibang and was not present at all in plant sample A of the same population.

Thus it is evident that the volatile oil of *A. afra* is remarkably variable in the major and minor oil compounds (Graven et al., 1992; Changonda et al., 1999; Dube, 2006; Viljoen et al., 2006b; Liu et al., 2009), with a large amount of monoterpenes and sesquiterpenes present (van Wyk and Wink, 2004). This variability is possibly due to the following factors: geographical variation; different plant parts used; drying methods; variation within the natural population (Liu et al., 2009)

A4 *Eucalyptus globulus* Labill.

Common names of *Eucalyptus globulus* Labill. are Eucalyptus (French); Blue gum; Tasmanian blue gum; true blue gum; impiskayihlangulwa, umdlavusa, umdlebe (Zulu); bloekom (Afrikaans); Eukalyptus (German); eucalipto (Italian) and eucalypto (Spanish) (Hutchings et al., 1996; van Wyk and Gericke, 2000; van Wyk and Wink, 2004; Suitor et al., 2009).

A4.1 Botanical description of *E. globulus*

E. globulus (Myrtaceae) is a very large tree (Figure A4.1). It has a characteristic shedding bark. The leaves are very glaucous, long and dimorphic as well as broad with solitary large white flowers (Figure A4.2) (Horn et al., 1964; van Wyk and Wink, 2004).

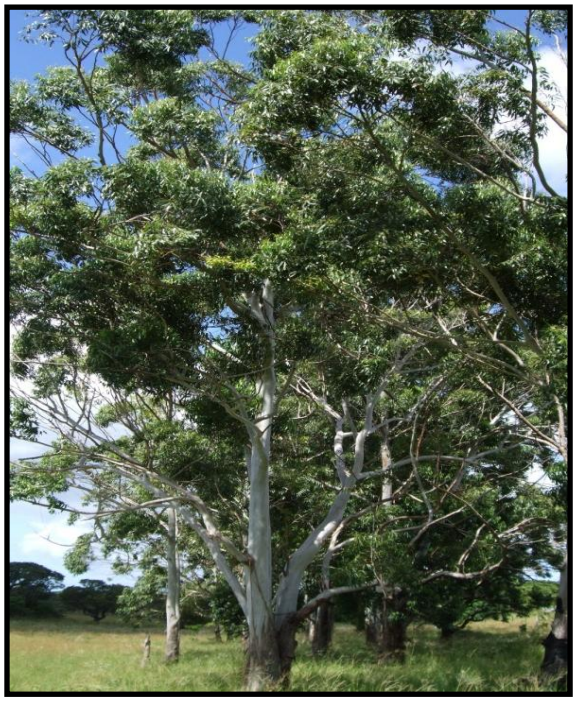


Figure A4.1 Large tree of *E. globulus* (S.F. van Vuuren).



Figure A4.2 Leaves and flowers of *E. globulus* (S.F. van Vuuren).

A4.2 Geographical locality of *E. globulus*

E. globulus is indigenous to Australia (Jordan et al., 1993; Dutkowski and Potts, 1999; van Wyk and Wink, 2004) and Tasmania. It is most widely cultivated in subtropical and Mediterranean regions (Takahashi et al., 2004). *E. globulus* was introduced into Europe, North and South Africa, California and the non-tropical districts of South America by Baron Ferdinand von Müller, a German botanist and explorer who made the qualities known all over the world (http://www.botanical.com/botanical/mgmh/e/eucaly14.html).

A4.3 Medicinal uses of *E. globulus*

Among the uses for *E. globulus* is as a spray to repel vermin. A poultice of *E. globulus* is used to draw out an abscess. The root is said to be a purgative. The leaf is used as a febrifuge and a remedy for leprosy. Leaf juice is used as a tonic and antiperiodic as well as an antiseptic (Watt and Breyer-Brandwijk, 1962). The essential oil is applied externally for the relief of rheumatism and minor skin ailments (van Wyk and Wink, 2004)

The main use of *E. globulus* in South Africa and elsewhere is as a remedy for respiratory complaints (such as colds and fever), and is used as an ingredient in nasal drops (Watt and Breyer-Brandwijk, 1962; Felhaber, 1997). The vapour from boiling the leaves is inhaled as a respiratory antiseptic as well as for diphtheria and croup. An infusion is drunk using a bunch of slightly boiled leaves in water or pouring boiling water over bruised leaves. This is used for the treatment of influenza. Inflammation of the lungs, chest pain and difficulty in breathing is treated by boiling a few leaves and then inhaling the vapours, which can induce sleep as well (Smith, 1895; Watt and Breyer-Brandwijk, 1962; van Wyk and Wink, 2004).

It is also reported by Caceres et al. (1991) to be used in the treatment of asthma, bronchitis, sore throat and chest tuberculosis, whooping cough and sinusitis. *E. globulus* is used for the treatment of coughs by acting as an expectorant (Mejía, 1927; McVaugh, 1963; Morton, 1981; Alvarez, 1987; Orellana, 1987 in Caceres et al., 1991). In a study conducted by Cacreres et al. (1991), *E. globulus* was proven effective in inhibiting the growth of *S. aureus* and *S. pyogenes.*

A4.4 Chemical constituents of *E. globulus*

E. globulus has been one of the most studied of *Eucalyptus* species and the essential oil has been well characterized (Singh et al., 2000; Batista-Pereira et al., 2006; Ghalem and Mohamed, 2008a). The main constituent that was found was 1,8-cineole (70-80%) (Stecher et al., 1968; van Wyk and Wink, 2004). Other compounds include α-pinene, phellandrene, terpineol, citronellal, geranyl acetate, eudesmol, eudesmyl acetate, piperitone, ρ-cymene and limonene, camphene, β-pinene, linalool, globulol and volatile aldehydes (isovaleric) were found (Stecher et al., 1968; Cimanga et al., 2002; van Wyk and Wink, 2004). Tohidpour et al. (2010) found eucalyptol, spathulenol, and α-pinene as the major constituents of *E. globulus*.

Major non-volatile compounds isolated from the ethanol extract of the leaves of *E. globulus* was identified as containing β-diletones, such as 16-hydroxy-18-tritriacontanone and 4-hydroxy-tritriacontane-16,18-dione (wax constituents), ellagic acid and its related compounds (Amakura et al., 2009). Other constituents include gallic acid, quercetin 3-*O*-β-D-glucuronide, globuluside, cryptmeriodol, 4-*epi*-cryptmeridiol, 3β,13β-dihydroxyurs-11-en-28-oic acid, β-eudesmol and macrocarpal I (Sakai et al., 2005). Amakura et al. (2009) identified the main constituents as gallic acid, oenothein, ellagic acid, quercetin 3-*O*-β-D-glucuronide, kaempferol 3-*O*-β-D-glucuronide and chlorogenic acid. Hasegawa et al. (2008) isolated two monoterpene conjugates of gallic acid, globulusin A and eucaglobulin from the hot water extract of *E. globulus*.

A5 *Leonotis. randii* S. Moore.

Leonotis microphylla is a synonym of *Leonotis ocymifolia* (Burm.f) Iwaarson var. *schinzii* (Gürke) according to Welman in Arnold and de Wet, 2002. *Leonotis randii* S. Moore. is the synonym used by Klopper et al., (2006). Common names include wild dagga (English); Knopdagga, wilde-dagga, Klipdagga (Afrikaans) which translates to "rock marijuana" (Vos, 1995) and semomonane (Watt and Breyer-Brandwijk, 1962).

A5.1 Botanical description of *L. randii*

Leonotis microphylla (Figure A5.1) is a perennial shrub (0.5-1m high) with densely covered stems, branches and leaves with long and short hispid hairs (Brown et al., 1912). It is a short plant displaying long flowering stems. Flowering of *L. randii* as shown in Figure A5.1 dominates in the summer rainfall season between October and February (Vos, 1995).



Figure A5.1 Flower of *L. randii* (S.F.van Vuuren).

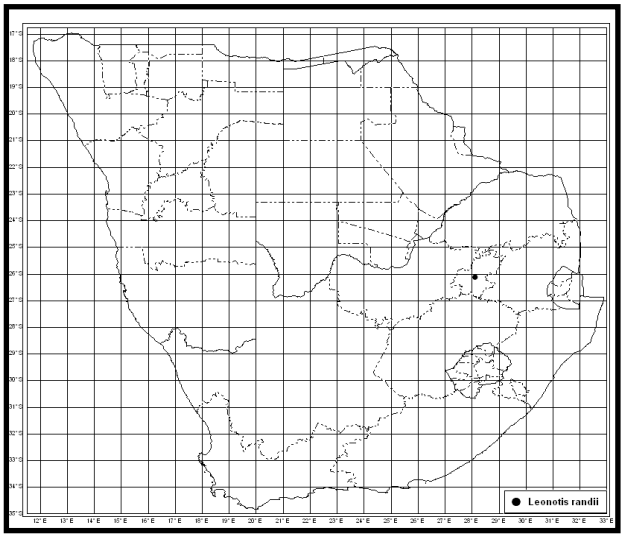


Figure A5.2 Distribution map of *L. randii* (SANBI).

A5.2 Geographical locality of *L. randii*

Its recorded distribution as shown in Figure A5.2 is in Gauteng (Transvaal), Free State (Orange Free State), and Botswana (Vos, 1995). It grows primarily in the Kalahari region of Gauteng (Transvaal), as shown in Figure A5.2, in the Jeppestown Ridges of Johannesburg, Meintjies Kop in Pretoria and in Heidelberg (Skan, 1910; Brown et al., 1912). Its habitat is restricted to bankenveld, sourveld, mixed bushveld, rocky outcrops and slopes (Vos, 1995).

A5.3 Medicinal uses of *L. randii*

The Africans use an infusion of *Leonotis microphylla* to apply to painful spots on the skin and haemorrhoids. The European and the Sotho people living in South Africa drink the infusion for the relief of digestive disturbances with fever as well as for chest affections. The Kwena, a branch of the Western Sotho people and the Tswana, Western Sotho people of South Africa drink a decoction of the plant in large doses for coughs and colds. The Manyika, people from the eastern part of Zimbabwe and across the border in Mozambique; a sub-tribe of the Shona, people in South Africa burn the flower of *L. microphylla* with porcupine quills and then inhale the smoke to stop nose bleeds (Watt and Breyer-Brandewijk, 1962; http://www.britannica.com/EBchecked/topic/325884/Kwena;
http://www.britannica.com/EBchecked/topic/607965/Tswana;
http://www.britannica.com/EBchecked/topic/363321/Manyika).

A5.4 Chemical constituents of *L. randii*

According to Vos (1995), 18 compounds were identified. The essential oil comprised of: caryophyllene 37%; germacrene 13%; eugenol 7%; phellandrene 3%; 1,8-cineole 2% and an unknown compound at retention time of 34.45 mins.

A6 *Lippia javanica* (Burm.f) Spreng

Synonyms of *Lippia javanica* are *Lantana galpiniana* Pearson, *Lippia asperifolia* Rich or *Verbena javanica* Burm. f. (Hutchings et al., 1996). Common names are fever tea, lemon bush (English); musukudu and bokhukhwane (Tswana); inzinziniba (Xhosa); umsuzwane, umswazi (Zulu); mumara (Shona); koors (tee) bossie, lemoenbossie, maagbossie, beukebos (Afrikaans); musudzungwane (Tshivenda) and lufisu (Nyakyusa) (Hutchings et al., 1996; Ngassapa et al., 2003; Manenzhe et al., 2004; Samie et al., 2005; van Wyk et al., 2009).

A6.1 Botanical description of *L. javanica*

L. javanica (Verbenaceae) is an erect woody shrub growing up to two meters in height. It has hairy leaves that have conspicuous veins (van Wyk et al., 2009). The leaf is described as being highly aromatic (van Wyk et al., 2009), having the odour of vanilla or mint (Watt and Breyer-Brandwijk, 1962), as well as having a strong lemon smell. Small yellowish white flowers (Figure A6.1) are produced in dense rounded heads (van Wyk et al., 2009).



Figure A6.1 Leaves of *L. javanica* with small yellowish white flowers (A.M. Viljoen.)

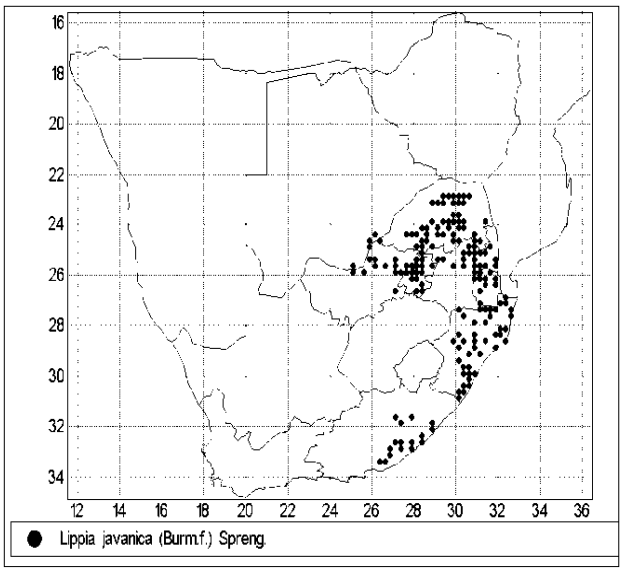


Figure 6.2 Geographical distribution of *L. javanica* in South Africa (SANBI).

A6.2 Geographical locality of *L. javanica*

L. javanica occurs over large parts of northern South Africa and it extends northward into tropical Africa (van Wyk et al., 2009) (Figure A6.2). Predominantly, it is found in the North-eastern regions of South Africa and extends into Botswana, Zimbabwe, Malawi and Swaziland (Chagonda et al., 1993; Manenzhe et al., 2004).

A6.3 Medicinal uses of *L. javanica*

L. javanica is predominantly used as an infusion for traditional medicine. Weak infusions are reportedly used as a caffeine-free tea substitute in some communities in Botswana (Watt and Breyer-Brandwijk, 1962; van Wyk et al., 2009; Shikanga et al., 2010; Manenzhe et al., 2004). Strong infusions are used topically to treat scabies and lice (van Wyk et al., 2009), it is also used as a calmative, anticolic, anticatarrh and as an emetic (Muchuweti et al., 2006). Pascual et al. (2001) documents *L. javanica* as being an analgesic, antipyretic, anti-inflammatory as well as being an antispasmodic and has antimicrobial and larvicidal/repellent properties (Mwangi et al., 1992; Hutchings and van Staden, 1994). It is also used to disinfect anthrax-infected meat (Watt and Breyer-Brandwijk, 1962). The Zulu speaking people from South Africa drink an infusion for the treatment of gangrenous rectitis and use the plant to treat measles, urticaria and other rashes (Watt and Breyer-Brandwijk, 1962; van Wyk et al., 2009. The cooled decoction is applied as a lotion to treat skin disorders such as rashes, stings and bites (van Wyk et al., 2009, Muchuweti et al., 2006). It is also stuffed in the nose as a remedy to stop nasal haemorrhage and colds, blackwater fever, malaria (Samie et al., 2005; Ngassapa et al., 2003), dysenteries e.g. diarrhoea (Samie et al., 2005), tapeworm infestations (Ngassapa et al., 2003), abdominal pains (Ngassapa et al., 2003) and other diseases (Watt and Breyer-Brandwijk, 1962). It is also used as a malaria remedy in South West Africa (Watt and Breyer-Brandwijk, 1962).

The Kwena and the Tswane people of South Africa use a decoction for the treatment of coughs and colds. Furthermore, the smoke from the burning plant is inhaled for respiratory conditions e.g. asthma and chronic cough (Samie et al., 2005; Muchuweti et al., 2006). The leaf boiled is in water and is used as a Swati (native people from South Africa and Swaziland) remedy for influenza and colds as well as a cough remedy by the Shangana (natives of Mozambique, and South Africa) (Watt and Breyer-Brandwijk, 1962). *L. javanica* leaves and stems are used by the Xhosa, natives of South Africa, as a weak infusion in milk or water for the treatment of coughs, colds and bronchial troubles, shortness of breath and chest complaints (Watt and Breyer-Brandwijk, 1962; Pascual et al., 2001). *L. javanica* is used traditionally to treat coughs, colds, bronchitis and various chest ailments. Hot leaf infusions are taken with milk or water.

A6.4 Chemical constituents of *L. javanica*

Non-volatile compounds Iridoid glucosides, theveside-NA and theviridoside, were isolated from *L. javanica* by Rimpler and Sauerbier (1986). The toxic triterpenoid, Icterogenin was isolated and reported by Watt and Breyer-Brandwijk (1962) and cited in Hutchings and van Staden (1994), Pascual et al. (2001), Buckingham (2006) and Mujovo et al. (2008).

Phenolic secondary metabolites have been recently studied by Olivier et al. (2010). Verbascoside and isoverbascoside, isolated from the aerial parts of *L. javanica*, are sugar moieties and thus render them water-soluble. They are well known for their biological activities (Herbert et al., 1991; Pieroni et al., 2000; Pennacchio, 2005) thus it said that the two compounds are probably linked to the medicinal uses of *L. javanica*. Traditional healers use the infusions of *L. javanica* in most cases, and thus extraction of these active constituents is most likely (Olivier et al., 2010).

The chemical composition of *L. javanica* essential oil has been thoroughly studied previously (Mwangi et al., 1992; Velasco-Negueruela et al., 1993; Hutchings and van Staden, 1994; Terblanche and Kornelius 1996; Ngassapa et al., 2003; Manenzhe et al., 2004; Viljoen et al., 2005). Neidlein and Staehle (1974) found caryophyllene, linalool and ρ-cymene as major components of *L. javanica* essential oil. Later on Mwangi et al. (1991) reported the presence of myrcenone, myrcene, (E) - and (Z) - tagetenone as the main characteristic components of the same. Terblanche and Kornelius (1996) in their review reported (Z)-tagetenone (24.9-39.9%), (E)-tagetenone(11.4-20.6%), myrcene (4.7-8.3%), myrcenone (20.9-49.7%) as the main constituents. β-caryophyllene was also present (0.1-2.0%) (Fujita, 1965; Mwangi et al., 1991; Velasco-Negueruela et al., 1993).

Samples of *L. javanica* essential oil from Tanzania were also analyzed by Balira (1986) and Ngassapa et al. (2003). Balira (1986) analyzed two samples, one from Kimbiji in Dares Salam (A), and the other from a southern highlands region in Tanzania Iringa (B). Sample A contained myrcenone in high amounts i.e. 52.2%. Sample B also contained myrcenone but at 9.6%. In the study by Ngassapa et al. (2003), two samples (A and B) were also chosen, but from the same location and they possessed different odours. Sample A contains limonene (9.6%), germacrene-D (9.0%), camphor (7.2%) and linalool (5.4%). Sample B had citral (geranial (21.5%) and neral (13.7%)) and limonene (11.3%). From this it is evident the two populations could be different chemotypes.

Manenzhe et al. (2004) sampled *L. javanica* essential oil in South Africa and found a major compound piperitone (74.4%), with other compounds at lower concentrations such as limonene (1.6%, linalool (1.8%), o-isopropenylanisole (9.7%), α-terpineol (0.8%), Z-Ocimenone (3.9%) and β-caryophyllene (1.0%). It was reported that the essential oil is highly variable, both quantitatively and qualitatively with variations present even in the same location (Chagonda et al., 2000). In 2005, Viljoen et al. performed a study on the geographical variation of *L. javanica* essential oil. Five natural populations each having 16 samples were collected i.e. Swaziland, Nelspruit, Warmbaths, Long Tom Pass and Fairlands. Five different chemotypes were identified within the different populations. They were 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%).

A7 *Osmitopsis asteriscoides* (L.) Cass.

Common names of *O. asteriscoides* include bellis, bels (e), belskruie (Afrikaans), and Mountain daisy (Bremer, 1972; Smith, 1966; van Wyk et al., 2009).

A7.1 Botanical description of *O. asteriscoides*

O. asteriscoides (Asteraceae) is a vigorous branched glabrous shrub growing up to two meters in height (Bremer, 1972). It is strongly camphor scented and has bare stems with its leaves crowded at the branch ends (van Wyk et al., 2009). The leaves are oblong and glandular displaying a large number of small glands on the surface (van Wyk et al., 2009). Due to these glands, this plant produces a high volume of essential oil. White flowers (Figure A7.1) bloom between January – December (whole year) (Bremer, 1972), and their heads are attractive and grouped on branch tips (van Wyk et al., 2009).



Figure A7.1 *O. asteriscoides* leaves and flowers (A.M. Viljoen).

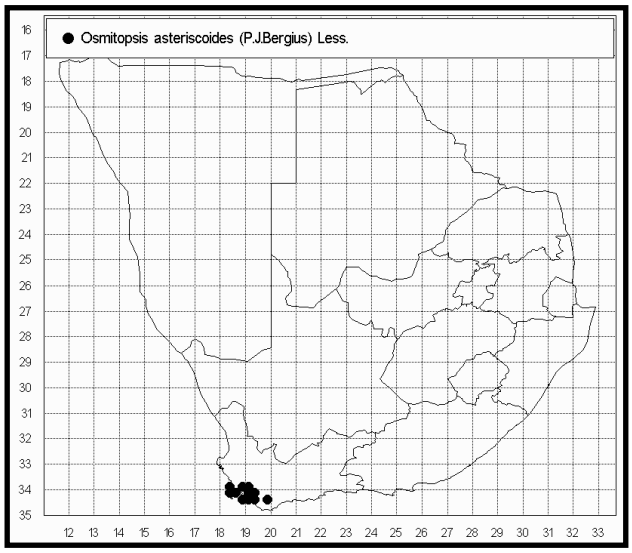


Figure A7.2 Geographical distribution of *O. asteriscoides* (SANBI).

A7.2 Geographical locality of *O. asteriscoides*

It is restricted to the Western Cape Province (Figure A7.2), where it thrives in open and moist ground but not in the shadow of trees (Bremer, 1972; Marloth, 1908). It then forms small "forests" and is said to be found in swampy places on the summit of Table Mountain.

A7.3 Medicinal uses of *O. asteriscoides*

O. asteriscoides is a traditional Cape Dutch remedy for the treatment of many ailments (van Wyk et al., 2009). Brandy tinctures (*Spiritus bellidis*) are used for coughs and hoarseness and the dried plant extract is applied externally for inflammation. A poultice of the leaf is applied

to cuts and swellings. It is very popular in the treatment of stomach troubles such as colic. Included in the spectrum of medicinal uses is the use for fever, influenza, body aches and pains, and as an embrocation in paralysis (Watt and Breyer-Brandwijk, 1962). According to Watt and Breyer-Brandewijk (1962), it is reported to cause sweating. Additionally, a tincture or an infusion is applied as a remedy for rheumatism (van Wyk et al., 2009; Viljoen et al., 2003; Watt and Breyer-Brandewijk, 1962).

A7.4 Chemical constituents of *O. asteriscoides*

The essential oil of *O. asteriscoides* is rich in 1, 8-cineole (eucalyptol) and camphor (van Wyk et al., 2009). It has been previously studied by Bohlmann and Zdero, (1974); Bohlman et al. (1985) and Viljoen et al. (2003). *O. asteriscoides* has been reported to have a high amount of non- volatile sesquitepene lactones including osmitopsin isolated from the leaves (Viljoen et al., 2003; McGaw et al., 2008).

A8 *Tetradenia riparia* (Hochst.) Codd

The common names of *Tetradenia riparia* include ginger bush (English); watersalie (Afrikaans); iboza and ibozane (Zulu) and umuravumba (Rwandanese) (Hutchings et al., 1996; van Wyk et al., 2009). Synonyms include *Iboza bainesii* N.E. Br., *Iboza galpinii* N.E. Br., *Iboza riparia* (Hochst.) N.E. Br., *Moschosma riparia* N.E. Br., and *Moschosma riparium* Hochst. (Hutchings et al., 1996).

A8.1 Botanical description of *T. riparia*

T. riparia (Hochst.) Codd belongs to the family Lamiaceae. It is a multi-branched shrub or a small tree (1-3 meters in height). The leaves and stems contain glandular hairs (Figure A8.1) with the leaves glandular on both surfaces (van Wyk et al., 2009). Male and female flowers (Figure A8.2), with colours ranging from white to mauve, are born on different plants. The female flower spikes are densely flowered and longer (up to 80mm long) compared to the male spikes (20m long) (Codd, 1985; van Wyk et al., 2009).



Figure A8.1 Glandular leaves of *T. riparia* (S.F. van Vuuren).



Figure A8.2 Flowers of *T. riparia* (H. de Wet).

A8.2 Geographical distribution of *T. riparia*

T. riparia is commonly found in the eastern parts of South Africa (Figure A8.3), on dry rocky slopes, wooded hillsides or on a stream bank in relatively frost-free areas (Codd, 1985; van Wyk et al., 2009). It extends into Namibia and Angola and then north into east tropical Africa and Ethiopia (Codd, 1985; van Wyk et al., 2009).

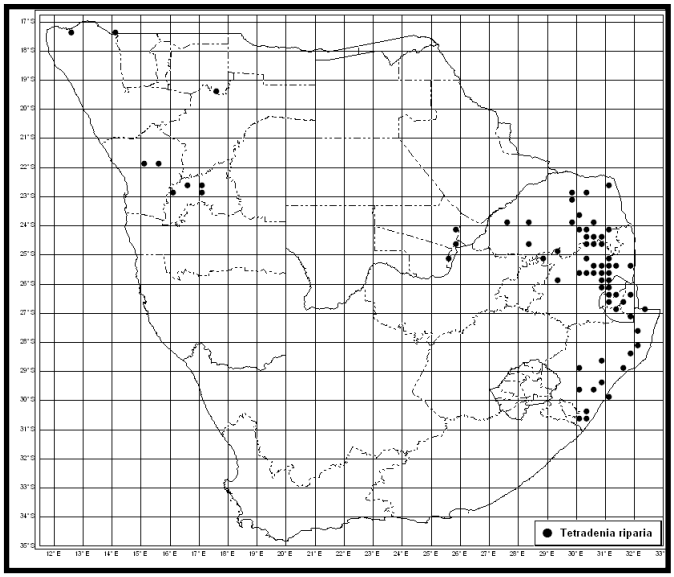


Figure A8.3 Botanical distribution of *T. riparia* (SANBI).

A8.3 Medicinal uses of *T. riparia*

T. riparia leaf infusions and decoctions are used for respiratory diseases such as coughs, colds, sore throat, and influenza as well as other ailments such as mouth ulcers, stomach-ache, diarrhoea, haemoptysis; boils, mumps fever, malaria, swollen legs (Watt and Breyer-Brandwijk, 1962; Hutchings, 1989; Pujol, 1990; Githinji and Kokwaro, 1993; Hutchings et al., 1996; van Wyk et al., 2009). Crushed leaves of *T. riparia* are also inhaled for the treatment of headaches (Hutchings and van Staden, 1994).

A8.4 Chemical constituents of *T. riparia*

The major essential oil components found in *T. riparia* were α-terpineol, fenchone, β-caryophyllene, and β-fenchyl alcohol (Campbell et al., 1997; Scott et al., 2004; van Wyk et al., 2009). Secondary chemical studies on *T. riparia* have revealed the presence of diterpenes which are ibozol and 7 α-hydroxyroyleanone; the diterpenediol i.e. 8(14),15-sandaracopimaradiene-7α,18-diol which possesses significant antimicrobial activity (De Kimpe et al., 1982); α-pyrones called umuravumbolide (5,6-dihydro-6-(3-acetoxy-1-heptenyl)-2-pyrone), tetradenolide (5,6-dihydro-6-(1,2-dihydroxyhexyl)-2-pyrone), deacetylmuravumbolide (5,6-dihydro-6-(3-hydroxyl-1-heptenyl)-2-pyrone) and deacetylboronolide (5,6-dihydro-6-(1,2,3-trihydroxyheptyl)-2-pyrone and sitosterol (Zelnik et al., 1978; van Puyvelde et al., 1979; van Puyvelde et al., 1986; van Puyvelde et al., 1987; Davies-Coleman and Rivett, 1995; Scott et al., 2004).

A9 *Zanthoxylum capense* (Thunb.) Harv.

Synonyms of *Zanthoxylum capense* include *Fagara capensis* Thunb., *F. Magaliesmontana* Engl., *Xanthoxylum* (*Xanthoxylon*) (Waterman, 1975) *capense* (Thunb.) Harv., *Z. thunbergii* DC. var *obtusifolia* Harv. (Hutchings et al., 1996). Common names include Small Knobwood, Adelaide Spice tree, and Cardamon. Other names include Wild cardamom (English); kleinperdepram, Paarde pram, kardamon, knop(pies)doring (Afrikaans); monokwane (Sotho); *umlungumabele* (Xhosa/Zulu); *umnungamabele, amabelentombi, amabelezintshingezi, isimungumabele, isinungwane, umnungwane omncane* (Zulu) (Smith, 1895; Hutchings et al., 1996; van Wyk et al., 2009).

A9.1 Botanical description of *Z. capense*

Z. capense (Thunb.) Harv. (Rutaceae) is a tree (about 5-10m in height) with many branches. Thick thorns on the bark are a characteristic trait of *Z. capense* trees. Sharp thorns are also present on the stems. Several pairs of leaflets are found on the leaf (Figure A9.1) with translucent oil glands dotted on the edges. The tree bears fruit, which is said to contain oil, in clusters, which are small and orange brown in colour and resemble oranges (Watt and Breyer-Brandwijk, 1962). The fruit is acrid and tastes of lemon, and presents with a persistent burning sensation in the mouth (Watt and Breyer-Brandwijk, 1962). When ripe, the fruits slit open and reveal shiny black seeds inside. The flowers that are born are greenish-white and are inconspicuous (van Wyk et al., 2009). Root and bark decoctions have a displeasing odour and are said to cause sweating (Watt and Breyer-Brandwijk, 1962; Hutchings et al., 1996).

A9.2 Geographical locality of *Z. capense*

Z. capense is distributed widely in the eastern and northern parts of South Africa (van Wyk et al., 2009). Distribution also extends sporadically into the southern regions (Figure A9.2).

A9.3 Medicinal uses of *Z. capense*

Z. capense has a reputation of being widely used as a medicine by the African and European people. An infusion of the leaf lends its uses for gastric (Hutchings et al., 1996) and intestinal disorders as well as intestinal parasites (Watt and Breyer-Brandwijk, 1962; Hutchings et al., 1996). The fruit has been used for flatulent colic, palsy, as a stomach remedy and for fever (Watt and Breyer-Brandwijk, 1962; Forbes, 1986; Hutchings et al., 1996; van Wyk et al., 2009). The bark is taken as a tonic in persons suffering from sores (Bryant, 1970; Hutchings

et al., 1996) as well as a snakebite treatment. The whole plant is burned on a hot plate and the fumes inhaled for dizziness (Oluwole et al., 2002).



Figure A9.1 Leaflets of *Z. capense* (S.F.van Vuuren).

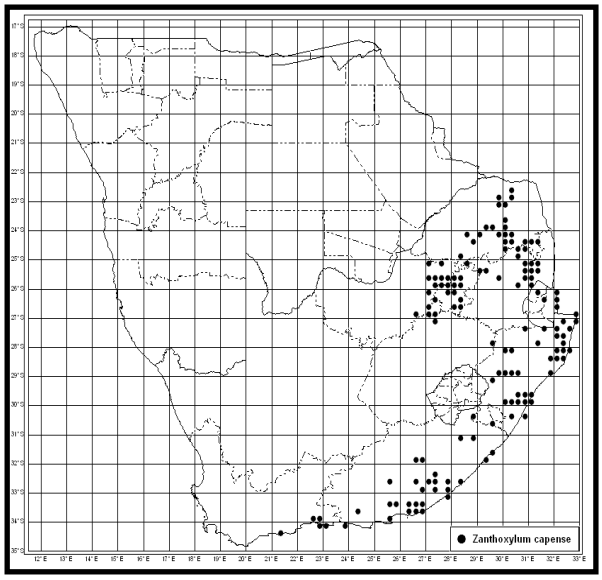


Figure A9.2 Geographical distribution of *Z. capense* in South Africa (SANBI).

A decoction of the bark is given to cattle for gall sickness. It is also used in combination with other medicinal plants to treat tuberculosis, paralysis, epilepsy, infertility and impotency, pleurisy, as an anthelminthic, a purgative, enema, parasiticide as well as for blood purifying (Watt and Breyer-Brandwijk, 1962; Watt, 1967; Hutchings et al., 1996; van Wyk et al., 2009).

A decoction of the root is used as a mouthwash (Watt and Breyer-Brandwijk, 1962; Pujol, 1990; Hutchings et al., 1996; Steyn et al., 1998; van Wyk et al., 2009) for aphthae in children and a lotion for acne. Powdered root taken by mouth has been used for pimples and blood poisoning. Powdered bark is used to treat paralysis and relieve toothache by loosening the tooth (Watt and Breyer-Brandwijk, 1962; Hutchings et al., 1996). The root bark as well as a paste of the inner stem bark is similarly used (Smith, 1895; Watt and Breyer-Brandwijk, 1962; Pujol, 1990; Hutchings et al., 1996; van Wyk et al., 2009). *Z. capense* is widely used to disinfect anthrax-infected meat as well as in the treatment of anthrax (Watt and Breyer-Brandwijk, 1962).

Other uses of the root of *Z capense* include the treatment of bronchitis and for violent chronic coughing when used in combination with other plants (Watt and Breyer-Brandwijk, 1962; Bryant, 1970; Hutchings et al., 1996; Steyn et al., 1998).

A9.4 Chemical constituents of *Z. capense*

In 1914, Juritz published the only reported chemical investigation at the time, of *Z. capense* and it was found that 'a large proportion of a resinous substance was extracted as well as tannins and a yellow colouring substance which was not identified' (Steyn et al., 1998). Since then, alkaloids from African *Zanthoxylum capense* were isolated (Calderwood et al., 1970; 1971 in Fish and Waterman, 1972). These are skimmianine, chelerythrine, nitidine and *N*-methyltetrahydropalmatine. In a study by Four compounds isolated from *Z. capense* were of interest, which were pellitorine, xanthoxylum-γ, γ-dimethylallyl ether, β-sitosterol and sitosterol-β-D-glucose (Steyn et al., 1998). The medicinal properties of *Z. capense* are said to be supported and explained by the presence of the secondary metabolites pellitorine and sitosterol-β-D-glucose (Steyn et al., 1998).

Appendix B – Conference/publication presentations

Publications/conference presentations arising from preliminary studies*

Co-author of the following poster: van Vuuren, S.F., Suliman, S., Dhorat, S. and AM Viljoen. The pharmacological interaction of commercial essential oils in combination with conventional antimicrobials. The 38th International Symposium on Essential Oils, Austria, Graz, 9-13 September, 2007.

Co-author of the following publication: van Vuuren, S.F., Suliman, S., and Viljoen, A.M., 2009. The antimicrobial activity of four commercial essential oils in combination with conventional antimicrobials. Letters in Applied Microbiology, 48 (4), 440-446.

Publications/conference presentations arising from this study*

Suliman, S.; van Vuuren, S.F.; Viljoen, A.M. 2008. Validating the antimicrobial efficacy of *Artemisia afra* in combination with medicinal plants to treat respiratory tract infections. Podium presentation, Academy of Pharmaceutical Sciences of South Africa (PSSA), 2008. Rustenburg, 22-26 September 2008. Young scientist competition presentation.

Suliman, S.; van Vuuren, S.F.; Viljoen, A.M. 2009. Antimicrobial interactions of *Artemisia afra* with other medicinal plants to treat respiratory diseases. Podium presentation, Annual Meeting of the Indigenous Plant Use Forum (IPUF). Stellenbosch, 6-9 July 2009. **3rd Prize winner of young scientist competition presentations**.

Suliman, S. van Vuuren, S.F.; Viljoen, A.M. 2009. The ethnobotanical study of *A. afra* in combination therapy for the treatment of respiratory tract infections. Podium presentation at the University of Johannesburg Post Graduated Symposium. Johannesburg, 29 October 2009.

Suliman, S., van Vuuren, S.F. and Viljoen, A.M., 2010. Validating the *in vitro* antimicrobial activity of *Artemisia afra* in polyherbal combinations to treat respiratory infections. South African Journal of Botany 76, 655-661.

*Abstracts can be found in Appendix C

Appendix C - Abstracts of presentations/conference presentations

THE PHARMACOLOGICAL INTERACTION OF COMMERCIAL ESSENTIAL OILS IN COMBINATION WITH CONVENTIONAL ANTIMICROBIALS

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ORIGINAL ARTICLE

The antimicrobial activity of four commercial essential oils in combination with conventional antimicrobials

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Keywords
antibiotic, essential oils, *Melaleuca alternifolia*, *Mentha piperita*, *Rosmarinus officinalis*, synergistic; additive; antagonistic; combination, *Thymus vulgaris*.

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2008/1252: received 21 July 2008, revised 15 October 2008 and accepted 17 November 2008

doi:10.1111/j.1472-765X.2008.02548.x

Abstract

Aims: Due to the emergence of multi-drug resistance, alternatives to conventional antimicrobial therapy are needed. This study aims to investigate the *in vitro* pharmacological interactions between essential oils (considered valuable as natural therapeutic treatments) and conventional antimicrobials (ciprofloxacin/amphotericin B) when used in combination.

Methods and Results: Interactions of the essential oils (*Melaleuca alternifolia*, *Thymus vulgaris*, *Mentha piperita* and *Rosmarinus officinalis*) when combined with ciprofloxacin against *Staphylococcus aureus* indicate mainly antagonistic profiles. When tested against *Klebsiella pneumoniae* the isobolograms show antagonistic, synergistic and additive interactions depending on the combined ratio. The *R. officinalis*/ciprofloxacin combination against *K. pneumoniae* displayed the most favourable synergistic pattern. The interactions of *M. alternifolia* (tea tree), *T. vulgaris* (thyme), *M. piperita* (peppermint) and *R. officinalis* (rosemary) essential oils with amphotericin B indicate mainly antagonistic profiles when tested against *Candida albicans*.

Conclusion: While a number of interactions show complete antagonism, others show varied (synergistic, additive and/or antagonistic) interactions, thus the efficacy is dependent on the ratio in which the two components co-exist.

Significance and Impact of the Study: The predominant antagonistic interactions noted here, suggests that some natural therapies containing essential oils should be used with caution when combined with antibiotics.

Validating the antimicrobial efficacy of *Artemisia afra* in combination with medicinal plants to treat respiratory tract infections

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Introduction:

Artemisia afra is one of the most widely used plants in South African traditional medicine. It is commonly used to treat respiratory infections like coughs, colds, lung inflammation and often combined with plants such as *Lippia javanica*, *Agathosma betulina*, *Osmitopsis asteriscoides*, *Eucalyptus globulus*, *Zanthoxylum capense*, *Leonotis microphylla*, *Tetradenia riparia* and *Allium sativum*. The aim of this study is to provide scientific validation on the synergistic, additive and possibly antagonistic interactions between *A. afra* plant combinations used in traditional medicinal practices.

Methods:

In vitro minimum inhibitory concentrations (MIC) assays were used to determine antimicrobial efficacy of the essential oil, dichloromethane: methanol (CH_2Cl_2: MeOH) and aqueous extracts. Calculation of fractional inhibitory concentrations (FIC) and isobologram studies were performed to determine pharmacodynamic interactions, such as synergy, antagonism or additive behaviour. Thin layer chromatography (TLC) combined with bio-autographic assays were used to visualise the antimicrobial activity.

Results:

MIC's results were plotted and isobolograms revealed that when *Artemisia afra* was combined with other plants, mostly additive and synergistic interactions was noted. The essential oil combinations mainly indicated additive interactions. With the aqueous extracts, synergy and antagonism was apparent. The most pronounced synergy was noted for the (CH_2Cl_2: MeOH) extract of *Artemisia afra* and *Eucalyptus globulus*. Synergy was observed

against *M. catarrhalis*, *K. pneumoniae*, *E. faecalis* and *C. neoformans*. TLC and bio-autographic assay results indicate several compounds responsible for antimicrobial activity.

Conclusion:

Results obtained from antimicrobial assays for various combinations of *Artemisia afra* with other plants (as prescribed by traditional healers) show varied interactions (synergy, antagonism and additive) depending on the selection of plant combination.

Antimicrobial interactions of *Artemisia afra* with other medicinal plants to treat respiratory diseases.

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Artemisia afra is one of the most widely used plants in South African traditional medicine. It is commonly used to treat respiratory infections such as coughs, colds, lung inflammation and often combined with plants such as *Lippia javanica*, *Agathosma betulina*, *Osmitopsis asteriscoides*, *Eucalyptus globulus*, *Zanthoxylum capense*, *Leonotis microphylla*, *Tetradenia riparia* and *Allium sativum*. The combinations were specifically analysed for any antimicrobial interactions. In addition, the combined use with milk, honey and salt, as traditionally prepared, was evaluated. Methods used included *in vitro* minimum inhibitory concentrations (MIC) assays, which were used to determine antimicrobial efficacy of the essential oil, dichloromethane: methanol and aqueous extracts. Calculation of fractional inhibitory concentrations (FIC) and isobologram studies were performed to determine pharmacological interactions. The most promising result was the combination of *A. afra* and *A. betulina* dichloromethane: methanol extract against *M. catarrhalis* with a FIC of 0.5. Isobologram results of this combination indicated synergistic, antagonistic and additive pharmacological behaviour against the four pathogens tested, i.e. *M. catarrhalis*, *E. faecalis*, *K. pneumoniae* and *C. neoformans*. Combinations of *A. afra* with honey, salt and milk showed mainly indifferent antimicrobial behaviour. An exception was noted i.e. *A. afra* (aqueous extract) combined with skim milk against *M. catarrhalis*, indicating additive behaviour with a FIC value of 0.63. Results obtained from the antimicrobial assays for various combinations of *A. afra* with other plants (as prescribed by traditional healers) show varied interactions depending on the selection of plant combination.

The ethnobotanical study of *A. afra* in combination therapy for the treatment of respiratory tract infections

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Artemisia afra is one of the most widely used medicinal plants in South Africa. It is commonly used to treat respiratory infections such as coughs, colds, lung inflammation and often combined with plants such as *Lippia javanica*, *Agathosma betulina*, *Osmitopsis asteriscoides*, *Eucalyptus globulus*, *Zanthoxylum capense*, *Leonotis microphylla*, *Tetradenia riparia* and *Allium sativum*. In addition, the combined use with milk, honey and salt is also traditionally prepared. These combinations were evaluated for their antimicrobial interactions. Methods used included *in vitro* minimum inhibitory concentrations (MIC) assays, which were used to determine antimicrobial efficacy of the essential oil, dichloromethane: methanol and aqueous extracts. Calculation of fractional inhibitory concentration (FIC) and isobolograms demonstrated pharmacological interactions. Results of the combinations of *A. afra* and *L. javanica*, and *A. afra* and *O. asteriscoides* essential oil, dichloromethane: methanol and aqueous extracts, showed mostly synergistic interactions against all our pathogens tested i.e. *M. catarrhalis*, *E. faecalis*, *K. pneumoniae* and *C. neoformans*. In the combination of *A. afra* and *A. betulina*, and *A. afra* and *Z. capense*, varied interactions were noted, with antagonism shown for the aqueous extracts, for all pathogens tested, depending on the ratio in which they were combined. When *A. afra* and *E. globulus* was combined mainly synergistic interactions was noted with all pathogens. Combinations of *A. afra* with honey, salt and milk showed mainly additive and indifferent antimicrobial behaviour. These antimicrobial interactive studies with *A. afra* demonstrate varied interactions depending on the selection of combination.

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South African Journal of Botany 76 (2010) 655–661
SOUTH AFRICAN JOURNAL OF BOTANY
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Validating the *in vitro* antimicrobial activity of *Artemisia afra* in polyherbal combinations to treat respiratory infections

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Received 29 March 2010; received in revised form 6 July 2010; accepted 14 July 2010

Abstract

Artemisia afra is one of the most widely used medicinal plants in African traditional medicine and is commonly administered in polyherbal combinations to treat respiratory infections. Focussing on plant volatiles, the aim of this study was to provide scientific evidence for the antimicrobial activity of *A. afra* (principle plant) in combination with essential oils from three medicinal aromatic plants; *Agathosma betulina*, *Eucalyptus globulus* and *Osmitopsis asteriscoides*. *In vitro* minimum inhibitory concentration (MIC) assays were undertaken on four pathogens (*Enterococcus faecalis* ATCC 29212, *Moraxella catarrhalis* ATCC 23246, *Klebsiella pneumoniae* NCTC 9633 and *Cryptococcus neoformans* ATCC 90112) to determine antimicrobial efficacy of the oils and their combinations. The fractional inhibitory concentration (FIC) and isobolograms were used to interpret pharmacodynamic interactions such as synergy, antagonism or additive profiles. The antimicrobial activity of the individual oils mostly displayed moderate activity. Predominantly, additive interactions were noted. The most prominent synergistic interaction (FIC value of 0.5) was observed when *A. afra* was combined with *O. asteriscoides* in the 8:2 ratio (eight parts *A. afra* with two parts *O. asteriscoides*) against *C. neoformans*. No antagonistic interactions were evident.
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Keywords: *Agathosma betulina*; *Artemisia afra*; Antimicrobial; *Eucalyptus globulus*; Essential oil; *Osmitopsis asteriscoides*; Synergistic and additive interactions