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SYNERGISTIC INTERACTIONS OF LAVENDER ESSENTIAL OIL



Stephanie de Rapper
April 2013

DECLARATION

I, Stephanie de Rapper, declare that this dissertation is my own work. It is being submitted in fulfilment 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.

Stephanie de Rapper **Date**

ABSTRACT

Essential oils are not only used singularly but have been used in combination for many years. There is, however, very little scientific evidence to support the claims made for combined antimicrobial efficacy. With this in mind, a study was designed to investigate the antimicrobial activity of Lavender (*Lavandula angustifolia*) essential oil, in combination with other essential oils with antimicrobial relevance.

The micro-dilution minimum inhibitory concentration (MIC) assay was undertaken, whereby the fractional inhibitory concentration (ΣFIC) was calculated for 54 oil combinations. When lavender oil was assayed in 1:1 ratios with other oils, synergistic (23.5%), additive (52.5%), non-interactive (23.5%) and antagonistic (0.5%) interactions were observed. Four 1:1 combinations were synergistic against *Candida albicans* and *Staphylococcus aureus* (*Lavandula angustifolia* in combination with *Daucus carota* (ΣFIC 0.50 and 0.50); *Juniperus virginiana* (ΣFIC 0.50 and 0.50); *Cinnamomum zeylanicum* (ΣFIC 0.40 and 0.50) and *Citrus sinensis* (ΣFIC 0.42 and 0.38)). In order to understand the antimicrobial potential of these synergistic essential oil combinations, further antimicrobial analysis was undertaken whereby the oils were placed in varying ratios. Two of the four combinations (*Lavandula angustifolia* in combination with either *Cinnamomum zeylanicum* or *Citrus sinensis*), were identified as the most promising, demonstrating synergy at varying ratios, and thus the major chemical constituents of the essential oils were investigated further.

The major chemical constituents identified in *Lavandula angustifolia* (GC-MS) were linalyl acetate (36.7%), linalool (31.4%) and terpinen-4-ol (14.9%). The GC-MS profiles for all other oils in the study were also confirmed. The major chemical constituents of the most promising essential oil combinations were investigated in equal and varying ratios to determine the effect of chemistry on antimicrobial outcome. When one of the major essential oil constituents (linalyl acetate) of *Lavandula angustifolia* was combined with limonene found in *Citrus sinensis*, synergistic interactions were noted for all nine combinations against *C. albicans*; including the ratio at which the two major constituents would be mixed should the two oils be combined.

Lavandula angustifolia essential oil was placed in combination with four conventional antimicrobial agents (ciprofloxacin, chloramphenicol, fusidic acid and nystatin) to determine which of these agents in combination with *Lavandula angustifolia* would demonstrate the best antimicrobial effect. Synergy was determined for *Lavandula angustifolia* in combination with ciprofloxacin against *S. aureus* (ΣFIC of 0.49) and *Lavandula angustifolia* in combination with chloramphenicol against *P. aeruginosa* (ΣFIC of 0.29). No antagonism was noted for the combinations investigated. When placed in variable ratios it was identified that *Lavandula angustifolia* provided the pivotal role in the synergistic interactions observed against *C. albicans* and *S. aureus*, with ratios higher in *Lavandula angustifolia* essential oil concentration showing considerably better antimicrobial effects.

In order to determine the antimicrobial effects of *Lavandula angustifolia* in triple essential oil combinations, the method of MODDE® Design of Experiments was employed. The Design of Experiments (MODDE 9.1®) software identified that *Lavandula angustifolia* (from the combination of *Lavandula angustifolia*: *Citrus sinensis*: *Cedrus atlantica*) and *Thymus vulgaris* (from the combination of *Lavandula angustifolia*: *Daucus carota*: *Thymus vulgaris*) were the essential oils with the greater antimicrobial effect in the combinations analysed.

Lavender remains one of the most sought after essential oils. This comprehensive study on the antimicrobial effects of Lavender (*Lavandula angustifolia*) combinations demonstrates promising *in vitro* effects and lends some credibility for combined use in aromatherapy for the treatment of infections.

DEDICATION

Deuteronomy 16: 15

"The Lord will bless the work of my hands and my joy will be complete."

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- I would like to take this opportunity to acknowledge that all wisdom and understanding come from My Heavenly Father and that it is by His grace and love that I am able to accomplish anything. Ephesians 3:20-21 "Now all glory to God, who is able, through His mighty power at work in us, to accomplish infinitely more than we ask or think. Glory to Him in the church and in Christ Jesus through all generations forever and ever! Amen."

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LIST OF PRESENTATIONS AND PUBLICATIONS ARISING FROM THIS STUDY

Presentations

Refer to Appendix A

1. De Rapper S., van Vuuren S.F., Kamatou G.P.P., Viljoen A.M. and Dagne E. The additive and synergistic antimicrobial effects of select frankincense and myrrh oils – a combination from the pharaonic pharmacopoeia. SAAB Conference – University of Pretoria, Gauteng, South Africa. January 15 -18, 2012.

2. De Rapper S., van Vuuren S.F., Kamatou G.P.P. and Viljoen A.M. The antimicrobial activity of *Lavandula angustifolia* essential oil in combination with other aroma-therapeutic oils. 33rd Annual Conference of the Academy of Pharmaceutical Sciences of South Africa- Rhodes University, Grahamstown, South Africa. September 12-15, 2012.

Publications

1. De Rapper S., van Vuuren S.F., Kamatou G.P.P., Viljoen A.M. and Dagne E. (2012) The additive and synergistic antimicrobial effects of select frankincense and myrrh oils – a combination from the pharaonic pharmacopoeia. *Letters in Applied Microbiology*, **54:** 352-358.

2. De Rapper S., van Vuuren S.F., Kamatou G.P.P. and Viljoen A.M. (2013) The antimicrobial activity of *Lavandula angustifolia* essential oil and use in combination with other essential oils. *Evidence-Based Complementary and Alternative Medicine*. Accepted April 8th, 2013. (doi: http://dx.doi.org/10.1155/2013/852049)

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LIST OF ABBREVIATIONS

%	Percent
% (v/v)	Percentage volume for volume
ΣFIC	The sum of the fractional inhibitory concentrations
µl	Microlitre
ATCC	American type culture collection
CAM	Complementary and alternative therapy
CFU	Colony forming units
CLSI	Clinical and Laboratory Standards Institute
DoE	Design of Experiments
DPMO	Deficits per million opportunities
FIC	Fractional inhibitory concentration
G	Grams
GC	Gas chromatography
GC-MS	Gas chromatography coupled to mass spectroscopy
H	Hours
INT	*p*-iodonitrotetrazolium
L	Litre
M	Metre
mg/ml	Milligram per millilitre
MGRSA	Methicillin and Gentamicin resistant *Staphylococcus aureus*
MIC	Minimum inhibitory concentration
Min	Minute
Ml	Millilitre
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass spectroscopy
NA	Not applicable
NCCLS	National Committee for Clinical Laboratory Standards
RRI	Relative retention index
Rt	Retention time
TSA	Tryptone Soya agar
TSB	Tryptone Soya broth
UTI	Urinary tract infection
WHO	World Health Organization
ZOI	Zone of inhibition

CHAPTER 1
GENERAL INTRODUCTION

1.1. Essential oil use in natural therapy

A study conducted by the World Health Organization (2004) estimated that 80% of people in developing countries, including South Africa make use of complementary and alternative therapies. Furthermore, The Natural Centre for Complementary and Alternative Medicine at the National Institutes of Health in America identified in a survey that in first world countries such as America, 36% of the population used complementary and alternative therapies (Arias, 2004).

A study conducted by Vincent and Furnham (1996) aimed to identify why people prefer complementary and alternative therapies over the use of orthodox, allopathic medicine. A questionnaire was used as a means of analysis for a test group of 250 people. Key factors were identified as to why people choose to use complementary and alternative medicines (CAM). Firstly, preference for CAM was based on the belief that the therapy is natural. Some of the test subjects also stated that they preferred CAM to that of allopathic medicine as it was effective in providing relief for their condition. While the last factor was found to be less important, people also responded that the cost-effectiveness of CAM was a deciding factor.

Essential oils, which form part of complementary and alternative therapy, have been defined by the Aromatherapy Organizations Council of the United Kingdom as "aromatic, volatile substances extracted by distillation or expression from a single botanical species. The resulting oils should have nothing added or removed" (Buckle, 2003). Essential oils are believed to be the most important part of a plant, the relationship compared to the importance of blood in the human body (Tisserand, 1988), however, very little is known of the value of this relationship. A variety of possible reasons have been acknowledged as to why plants produce essential oils. Essential oils are thought to be produced in order to repel insects and herbivores from attack as well as to prevent bacterial and fungal infection. Essential oils are also believed to attract bees and insects for plant pollination while acting as a natural medicine for the treatment of plant wounds. Lastly, essential oils are also believed to be produced by plants in order to aid in survival where growth conditions are challenging (Price

and Price, 1992). The essential oil of a plant is produced from various regions such as the leaf, root, fruit or flower, and stored in specialized cells known as trichomes, scales or reservoirs or in some cases intracellular spaces of a plant (Worwood, 1990). Once identified, this part of the plant is harvested and the essential oil isolated.

Steam distillation is the most commonly employed method for obtaining essential oils worldwide (Başer and Buchbauer, 2010) (Figure 1.1). The plant material is placed in a large container with a small quantity of water. This container is then heated, and as a result the water in the container begins to boil. The boiled water breaks down the plant material, releasing the plants' essential oils. Due to the heat, the essential oils are then vaporized together with the steam of the heated water. The vaporized oil and water is then forced into a jacket known as a condenser, which contains cold, flowing water, which cools and condenses the vapours into a liquid state. The cooled liquid then drips back into a receiver, where the oil and water separate due to variances in density. The essential oil can then be collected and filtered for use (Curtis, 1996).

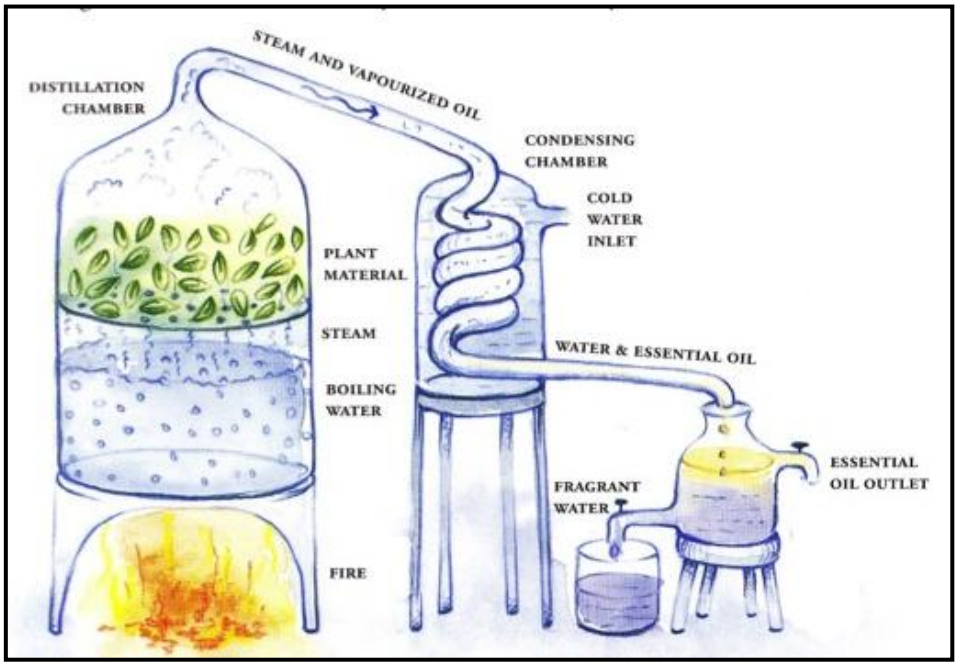


Figure 1.1 Schematic indication of the process of steam distillation (www.attunearoma.com).

The production of an essential oil takes a great deal of work and time as only small amounts of essential oil are produced from large quantities of raw plant material. For example, rose

essential oil requires 60 000 blossoms to produce 28.35 g of essential oil, while 100 kg of plant material is required to produce 3 kg of lavender essential oil (Worwood, 1990).

1.2. History of essential oil use

Essential oils have been used as part of herbal practice for their therapeutic and fragrant properties for as long as 60 000 years (Erichsen-Brown, 1979). The art of using essential oils for therapeutic practice has an extensive history reaching many continents around the world (Buckle, 2003). The earliest use of essential oils can be traced back to Iraq where archaeologists discovered plant material near a Neanderthal skeleton. This plant material included Yarrow which is known as an aromatic herb for the production of essential oils (Buckle, 2003).

The Chinese are considered the earlier founders of the therapeutic properties of essential oils with a Chinese herbal manuscript (The Great Herbal Pen Ts'ao) written in 2800 BC. In this manuscript 350 plant sources are indicated for the treatment of certain disease states (Buckle, 2003). In 2000 BC the Chinese Emperor produced a text titled "The Yellow Emperors Classic of Internal Medicine".

In India, the earliest writings confirming the use of essential oils for therapeutic purposes date back to 2000 BC. These writings called "Caraka Samhita" and "Sushrata Sambita" formed the basis of Ayurvedic medicine. These writings included over 700 plants for therapeutic purposes including ginger, coriander, cinnamon, sandalwood and myrrh (Swerdlow, 2000).

Greek history contains large sources of writings dating back to 300 BC, where essential oils are specified for therapeutic use. In 300 BC a Greek doctor by the name of Theophrastus wrote a manuscript entitled "Enquiry into Plants", where he indicated the use of essential oils for therapeutic purposes (Buckle, 2003). Hippocrates, a Greek doctor known as the 'father of medicine' understood the power of essential oils and often prescribed them to patients. A famous preparation prescribed by Hippocrates known as 'megaleion' contained myrrh, cinnamon and cassis for the treatment of skin wounds (Lawless, 1995). Hippocrates is also famously quoted for having said, "The way to health is to have an aromatic bath and scented massage every day" (Worwood, 1990). Around 100 AD, the Greek herbalist Pedanios

Dioskurides wrote the book entitled "De Materia Medica" in which 700 plants were illustrated for their therapeutic use. The most famous example of a plant stated in Pedanios Dioscurides work was the plant tarragon, which was cited for the use in cancer, gangrene, abortions and protection against snake bites (Buckle, 2003).

In 1347 the bubonic plague, also known as the Black Death, affected England, and it is believed that during this time that the English town of Bucklesbury survived the plague due to its large trade in lavender (Deinenger, 1995). Glove makers and perfumers in Europe were thought to survive due to the use of essential oils in their trade. Perfumes were created from essential oils while the material used to create gloves included an amount of essential oil (Buckle, 2003). During this period, it has been suggested that Nostradamus was successful in his treatment of plague victims with tablets containing crushed roses (Guenther, 1952).

The earliest scientific encounter of the use of essential oils is dated to the early 1920s where the French chemist René-Maurice Gattefosse experienced a terrible accident in his laboratory which caused burns to his arm. In a state of panic it is believed that Gattefosse shoved his arm into the nearest canister containing a cooling liquid which was later discovered to be lavender essential oil. The lesions on Gattefosse's arm healed quickly leaving no scar and it was due to this reaction that Gattefosse aimed to identify the healing and therapeutic properties of essential oils (Worwood, 1990).

By the 13thcentury, the doctor Arnald de Villanova first prescribed the use of essential oils in therapeutic practice and by the 17th century essential oils were applied more commonly by pharmacists who would advise the use of these oils primarily in topical applications (Hili, 2001).

1.3. Modern uses of essential oils

The practice of aromatherapy and the use of essential oils for therapeutic purposes has maintained its popularity through the ages with the practice continued today. Essential oils, applied as an alternate therapy, are widely known for their antimicrobial properties, which have been found to be beneficial in practical use in the fields of dermatology, gastritis, respiratory complaints, wound healing and genital infections. This increased interest in

aromatherapy has launched essential oils into the international markets, where they are often sold as ‘natural antibiotics’ (Hili, 2001; van Vuuren, 2008).

In the United Kingdom aromatherapy is used for the treatment of a plethora of conditions including pain and discomfort relief, infections, wound healing, burns, constipation and stress (Micozzi, 1996). In the South African context, aromatherapy is commonly employed in hospital and hospice settings with therapists attending to patients in Groote Schuur Hospital and St Luke’s Hospice, both situated in Cape Town. The essential oils most commonly used include lavender, frankincense, benzoin and rose otto in the treatment of general aches and pains, muscle tenderness and oedema (Price and Price, 1992). A study conducted by Diedericks (2006) aimed to identify a possible means of pain suppression in paediatric patients in a hospital setting. Diedericks suggested that when treating paediatric patients, a holistic approach be used. The incorporation of medication and complementary therapies, such as aromatherapy, was identified as obtaining the best result for paediatric pain suppression.

Essential oils, such as lavender, are used extensively due to its increased popularity attributed to the apparent calmative and relaxing activity on the mind and soul, as well as antimicrobial activity. Many products are currently being marketed with lavender as a primary or major ingredient in many industrial fields (Department of Agriculture, Forestry and Fisheries South Africa, 2009).

Other essential oils such as peppermint, thyme and clove have been used in industries due to their antimicrobial and preservative properties (Nakatsu *et al*., 2000; Halcon, 2002). A study conducted by Hasan (1994) aimed to identify the antimicrobial effects of five essential spice oils for the employment in food preservation. It was identified that the spice oils reduced fungal growth at a concentration of 0.1%, while complete inhibition of fungi was noted for the oil of cinnamon at a concentration of 1.0%. Another study conducted by Shaaya *et al*. (1997) further proved the efficacy of essential oils as antimicrobial agents for food preservation by investigating the oils of basil, anise and oregano, with positive inhibitory effects identified. Coca-Cola is the most popular product in which essential oils are employed. The secret formula to its flavour is made of essential oil blends. The secret “7x recipe” includes 20 drops of orange essential oil, 30 drops of lemon oil, 10 drops of nutmeg

oil, 5 drops of coriander oil, 10 drops of niaouli oil and 10 drops of cinnamon oil (Bates, 2011).

1.4. Route of administration of essential oils

Essential oils are typically administered by three means; topically, internally or via inhalation. For topical administration, essential oils are easily absorbed by the skin due to their lipophilic character. The skin acts as a barrier with substances richer in fats being absorbed more easily (Buckle, 2003). The greater the area of skin covered in essential oil, the greater the absorption will be (Balacs, 1993). Occlusions and dressings are suggested for use in this application as these oils are subject to evaporation, resulting in loss of essential oil for absorption (Buckle, 2003). Many factors may impact on the absorption of essential oils by the skin namely; intrinisic factors such as the thickness of the dermal layer, the area of skin, the presence of reservoirs and enzymes. Other factors include the hydration of the skin, viscosity of the essential oil and the molecular size of the compounds in an essential oil (Price and Price, 1992). This means of administration can be undertaken via massage, baths and hot and cold compresses (McGilvery and Reed, 1995). The topical application of essential oils is indicated in the treatment of skin infections and topical inflammation.

A study conducted by Fuchs *et al*. (1997) identified that carvone, a major chemical compound found in spearmint and caraway oils, was identified in a test subjects bloodstream after 10 minutes of essential oil massage. Another study conducted by Jäger (1992), identified lavender essential oils major chemical constituents in the bloodstream of a test subject after 10 minutes of essential oil massage. These results suggest that essential oils are quickly and easily made systemically available for therapeutic effect, via topical application.

The internal administration of essential oils is not well documented and the practice of this route of administration is not recommended (Buckle, 2003). When attempting this mode of administration, it is suggested that the practitioner knows the maximum daily allowance of a particular essential oil, as some components of these oils are found to be toxic. It has been recommended that no more than three drops of an essential oil, three times a day for three weeks be used (Price and Price, 1992). Internal administration of essential oils is suggested in the treatment of gastrointestinal complaints, cystitis or in the treatment of oral mucosal

infections and lesions. Essential oils can be delivered to the system internally by means of douches, mouthwashes or gelatine capsules (Buckle, 2003).

Inhalation is the safest and fastest means for the delivery of essential oil to the body's system. This is due to the large surface area provided by the alveoli in the lungs. The alveoli contain a large circulatory system resulting in quick diffusion of essential oil molecules to the bloodstream for therapeutic effect. The inhalation of essential oils works synergistically with the limbic system of the brain resulting in greater therapeutic effects (Buckle, 2003). The limbic system comprises of the amygdala and the hippocampus which are responsible for hormonal releases, resulting in sedative and relaxing responses of the body (Price and Price, 1992). This means of administration can be undertaken either directly in the case of an individual patient or indirectly for the treatment of a larger group of people. The direct method of essential oil inhalation includes the use of steam, oxygen-therapy hood or by means of cotton wool containing essential oil placed around the nose for inhalation. The indirect method of essential oil inhalation uses machinery such as room fresheners, burners, fans, humidifiers, diffusers, nebulizers and spritzer sprays to deliver large amounts of essential oil to a room for inhalation. This means of essential oil administration is indicated in the treatment of hayfever, asthma as well as upper and lower respiratory tract infections (Buckle, 2003).

1.5. Essential oils for use in combination

Essential oils are typically used in combination due to their synergistic and quenching effects for therapeutic purposes (Hall, 1904). Essential oil synergy relates to the use of more than one essential oil to bring about a greater means of activity than would be achieved if an essential oil was used individually (Price and Price, 1992).

In Tibetan medicine aromatherapy is cited for the treatment of disease states where combinations of essential oils are primarily used. These remedies are thought to have been created during pre-Buddist times forming part of the eighth century. One such preparation includes the use of clove, cardamom, sandalwood and myrrh for inhalation purposes (Buckle, 2003).

The use of essential oil combinations have also been accepted in medical practice. Hospitals in America have employed the use of essential oil blends in their operating rooms in order to reduce bacterial load and cross-contamination (Buckle, 2003). In the pharmaceutical sector in recent years many patents have been granted for the marketing of products containing essential oils as major ingredients in formulations. A study was conducted by Thacharodi and Rao (1994) in which the aim was to identify the effects of co-administration of essential oils with nifedipine, a drug used to reduce high blood pressure. It was identified that when the essential oils of ylang-ylang, lavender or cinnamon were added to an oil-water emulsion containing nifedipine, the penetration through the skin was greatly enhanced.

Many books on essential oil combinations have been written with numerous blends and combinations identified. Appendix B indicates over 600 possible essential oil combinations identified (Sellar, 1992; Lawless, 1995; Curtis, 1996; Shealy, 1998; Hili, 2001; Buckle, 2003; Lawrence, 2005). Although these texts have been published citing the use of these essential oils in combination for antimicrobial purposes, very few of these combinations have been validated by scientific studies. When searching for scientific reports using internet tools such as Pubmed, ScienceDirect and Scopus, a large void in research was evident for combination studies on essential oils.

In the field of aromatherapy, essential oils are very seldom used individually, but rather blended for a presumed enhanced therapeutic effect. With this in mind, a study was designed with the aim of identifying the antimicrobial activity of (*Lavandula angustifolia*) and essential oil combinations (Appendix B) where combination therapy is proposed. The reason why *L. angustifolia* was selected as the focus essential oil for further study was that upon analysis of the essential oil combinations (Appendix B) lavender was observed as the essential oil to be most frequently used in combination therapy (Table 1.1).

Table 1.1 Summary of the number of possible combinations (Appendix B) cited per essential oil for antimicrobial purposes in aromatherapy.

Essential oil		Frequency of citation
Common name	**Scientific name**	
Lavender	*Lavandula angustifolia*	43
Lemon	*Citrus medica limonum*	37
Orange	*Citrus sinensis*	34
Geranium	*Pelargonium odoratissimum*	32
Rosemary	*Rosmarinus officinalis*	29
Sandalwood	*Santalum album*	28
Cedarwood	*Juniperus virginiana*	27
Sage	*Salvia sclarea*	22
Ylang-Ylang	*Cananga odorata*	21
Cypress	*Cupressus sempervirens*	19
Tea-tree	*Melaleuca alternifolia*	19
Basil	*Ocimum basilicum*	17
Chamomile	*Anthemis nobilis*	17
Thyme	*Thymus vulgaris*	17
Clove	*Eugenia caryophyllus*	16
Palmarosa	*Cymbopogon martini*	16
Benzoin	*Styrax benzoin*	15
Coriander	*Coriandrum sativum*	15
Ginger	*Zingiber officinale*	15
Pine	*Pinus sylvestris*	15
Rosewood	*Dalbergia nigra*	15
Eucalyptus	*Eucalyptus globulus*	14
Pettitgrain	*Citrus aurantium*	13
Vetivert	*Andropogon muricatus*	12
Patchouli	*Pogostemon patchouli*	11
Peppermint	*Mentha piperita*	11
Marjoram	*Origanum marjorana*	11
Black pepper	*Piper nigrum*	10
Cinnamon	*Cinnamomum zeylanicum*	10
Lemongrass	*Cymbopogon citrates*	10
Grapefruit	*Citrus grandis*	9
Citronella	*Cymbopogon nardus*	8
Cardamom	*Elettaria cardamomum*	7
Myrrh	*Commiphora myrrha*	7
Fennel	*Foeniculum dulce*	6
Myrtle	*Myrtus communis*	6
Angelica	*Angelica archangelica*	5
Bay	*Laurus nobilis*	5
Niaouli	*Melaleuca viridiflora*	4
Caraway	*Carum carvi*	3
Elemi	*Canarium luzonicum*	3
Carrot seed	*Daucus carota*	2
Nutmeg	*Myristica fragrans*	2
May chang	*Litsea cubeba*	1
Fir	*Abies balsamea*	1
Tarragon	*Artemisia dracunculus*	1

1.6. The use of *Lavandula angustifolia* essential oil in the field of aromatherapy

L. angustifolia is considered the most versatile and popular of essential oils used in aromatherapy due to its apparent lack of toxicity (Curtis, 1996), and is applied primarily in massage (Chu and Kemper, 2001). Its therapeutic use could be traced back to as early as Roman and Greek times. *L. angustifolia* essential oil is believed to be of immense value for many skin conditions as it promotes growth of new skin as well as balancing its sebum production (Sellar, 1992). *L. angustifolia* is indicated in the treatment of burns (including sunburn), acne, eczema, psoriasis, abcesses, boils, carbuncles, fungal wounds, scarring, gangrene, insect bites, stings, dermatitis, rosacea, allergies and dandruff (Sellar, 1992; Lawless, 1995; Curtis, 1996). In combination with *Citrus medica limonum*, *L. angustifolia* is said to be particularly effective in the treatment of boils when used as a compress (Curtis, 1996). *L. angustifolia* essential oil use is also indicated for respiratory system conditions such as for the treatment of asthma, bronchitis, halitosis, catarrh, laryngitis, whooping cough and the common cold (Sellar, 1992; Lawless, 1995). When applied to bath water, *L. angustifolia* essential oil is found to be beneficial in the treatment of cystitis, dysmenorrhoea and leucorrhoea (Sellar, 1992; Lawless, 1995).

1.7. The use of *Lavandula angustifolia* essential oil for antimicrobial purposes

The specific use of *L. angustifolia* in combination with various other essential oils is given in Appendix C with specific reference to combined antimicrobial activity. The antimicrobial effects of *L. angustifolia* essential oil have been investigated for many years against a variety of micro-organisms using a diversity of testing methods. Some of these studies are summarised in Table 1.2. These studies have indicated the potential of *L. angustifolia* essential oil as a promising antimicrobial agent.

Table 1.2 Previous studies conducted on the antimicrobial effects of *L. angustifolia* essential oil.

Origin	Source	Bioactivity	Highest activity	Method	Best activity	Reference
Greece	NS*	Antifungal	*Trichophyton rubrum* and *Trichosporon beigelii*	ZOI**	*T. rubrum* - 40 mm	Adam *et al*., 1998
Australia	Flower	Antifungal Antibacterial	*Candida albicans* and *Acinetobacter baumannii*	MIC***	*C. albicans* and *E. coli* - 0.50% (v/v)	Hammer *et al.*, 1998
Australia	Flower	Antifungal Antibacterial	*C. albicans, Staphylococcus aureus* and *Escherichia coli*	MIC	*E. coli* - 0.25% (v/v)	Hammer *et al*., 1999
NS	NS	Antifungal Antibacterial	*E. coli* and *C. albicans*	MIC	*C. albicans* - 11.27 µl/ml	Giordani *et al.*, 2004
NS	Flower	Antifungal	*Trichophyton erinacei*, *T. mentagophytes, T. rubrum*, *T. shoeleinii*, *T. soudanense*, *T. tonsurans*	MIC	*T. soudanense* - 0.25 mg/ml	Shin and Lim, 2004
India	NS	Antifungal	*Aspergillus niger*	ZOI	*A. niger* - 15mm	Pawar and Thaker, 2006.
USA	NS	Antibacterial	Methicillin resistant *Staphylococcus aureus* (MRSA)	ZOI	MRSA - 26 mm	Chao *et al*., 2008
Belgium	NS	Antifungal Antibacterial	*Pasturella multocida, Bordetella bronchiseptica* and *Aeromonas hydrophila*	MIC	*P. multifocida* - 0.27% (v/v)	Mayaud *et al.*, 2008
USA	NS	Antifungal Antibacterial	*S. mutans*, *P. gingivalis, C. albicans*	ZOI	*S. mutans* - 2.40 mm	Sterer *et al*., 2008
Romania	NS	Antibacterial	*S. aureus*	ZOI	*S. aureus* - 6.25 mm	Fit *et al*., 2009
France	NS	Antifungal Antibacterial	*S. aureus, Staphylococcus epidermidis*, *C. albicans*	ZOI	*S. aureus* - 12 mm	Warnke *et al*., 2009
India	NS	Antifungal	*C. albicans*	ZOI	*C. albicans* - 1.20 mm	Agarwal *et al*., 2010
Morocco	Flower	Antifungal	*L. monocytogenes*	ZOI	*L. monocytogenes* - 15 mm	Bayoub *et al.*, 2010
Serbia	NS	Antibacterial	*Bacillus subtilus, Staphylococcus epidermidis, S. aureus, Salmonella entiriditis, Salmonella typhi, E. coli, Enterobacter cloacae.*	ZOI	*B. flavus* - 22 mm	Soković *et al.*, 2010
Jordan	Flower	Antifungal Antibacterial	*S. aureus*, MRSA, *Bacillus subtilus*, *Bacilus cereus, Klebsiella pneumoniae*, *S. typhi, E. coli*, *P. aeruginosa, C. violaceum, C. albicans, Candida glabrata*.	ZOI	*C. violaceum* - 7 mm	Al-Hussaini and Mahasneh, 2011
Cairo	Flower	Antibacterial	*L. innocua*, *S. marcescens, P. fluorescens*	ZOI	*L. innocua* - 15 mm	Viuda-Martos, *et al*., 2011

*NS – Not specified, **ZOI – Zone of Inhibition assay, ***MIC – Minimum Inhibitory Concentration assay

Upon investigation of *L. angustifolia* essential oils indicated uses, multiple essential oils were discovered in which *L. angustifolia* is combined for aroma-therapeutic purposes (Appendix C), however no scientific studies have been conducted to validate these claims. While, the antimicrobial activity of *L. angustifolia* essential oil has been studied extensively, the predominant method used (disc diffusion) to identify this activity has been found to be ineffective for the analysis of essential oils. The high viscosity and hydrophobicity of essential oils results in an inability to correctly dilute the test sample in the medium resulting in an unequal distribution of the oil (Kalemba and Kunicka, 2003). Additionally, essential oils are very complex mixtures of compounds which during incubation result in the loss of sample due to evaporation. This reasoning for the lack of accuracy for determination of antimicrobial effects of essential oils by means of disc diffusion analysis has been supported by a number of publications (Janssen *et al.*, 1987; Pauli and Kubeczka, 1997; Rios and Recio, 2005; Cos *et al.*, 2006). The MIC method used for analysis of the antimicrobial activity of essential oils involves the contact of a micro-organism to serial dilutions of the test sample (van Vuuren, 2008). This method of MIC determination is considered the preferred means of analysis for essential oils (Kalemba and Kunicka, 2003).

1.8. Study aims and objectives

The aim of this study was to investigate the antimicrobial activity of *L. angustifolia* in various essential oil combinations. Furthermore, the essential oil chemistry and its role in the antimicrobial outcome was investigated. A further breakdown of the specific objectives of this study is as follows;

1.8.1. To determine the chemical composition of *L. angustifolia* and 54 other essential oils used in this study by means of gas chromatography coupled with mass spectrometry (GC-MS) apparatus.

1.8.2. To determine the antimicrobial activity of *L. angustifolia* and commonly combined aroma-therapeutic essential oils using the MIC assay.

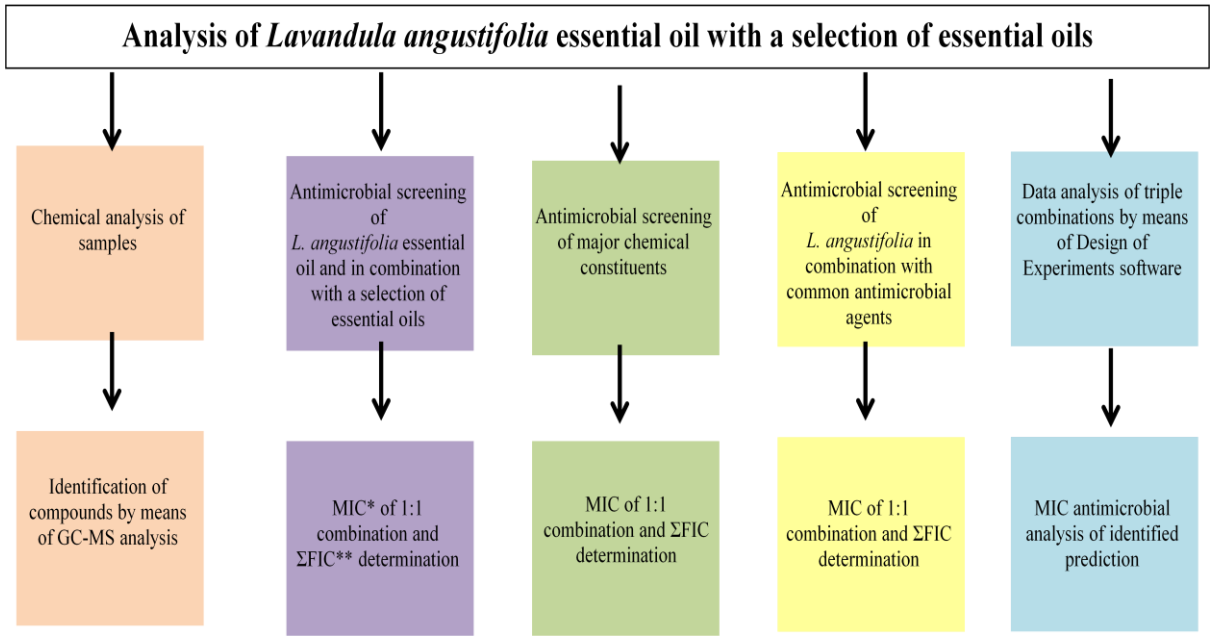
1.8.3. To determine the antimicrobial interaction of *L. angustifolia* essential oil with 54 essential oils at 1:1 concentrations (calculation of the fractional inhibitory concentration ΣFIC) and at various concentration combinations (isobolgrams).

1.8.4. To determine the antimicrobial activity of the major chemical constituents of the most promising *L. angustifolia* essential oil combinations at equal and varying ratios.

1.8.5. To determine the antimicrobial activity of *L. angustifolia* essential oil in combination with common conventional antimicrobial agents.

1.8.6. To optimise triple antimicrobial combinations including *L. angustifolia*, by using the Design of Experiments Software, MODDE 9.1®.

A flow chart (Figure 1.2) presents how each study was systematically followed.



* MIC – Minimum Inhibitory Concentration, ** FIC – Fractional Inhibitory Concentration

Figure 1.2 A schematic outlay of the study for the antimicrobial investigation of *L. angustifolia* with other oils of therapeutic importance.

CHAPTER 2
MATERIALS AND METHODS

2.1. Essential oil selection

Fifty-four essential oil samples were obtained from commercial fragrance and flavour suppliers (Table 2.1). These suppliers include Robertet (France), Givaudan (Switzerland) and Clive Teubes cc (South Africa). All oils were confirmed unadulterated and supplied with a certificate of analysis.

Table 2.1 The fity-four essential oils obtained from commercial suppliers for use in combination with *L. angustifolia*.

Scientific name	Common name
Abies balsamea	Fir
Andropogon muricatus	Vetiver
Angelica archangelica (root)	Angelica
Angelica archangelica (seed)	Angelica
Anthemis nobilis	Chamomile
Artemisia dracunculus	Tarragon
Canarium luzonicum	Elemi
Cananga odorata (heads)	Ylang-Ylang
Cananga odorata	Ylang-Ylang
Carum carvi	Caraway
Cedrus atlantica	Cedarwood
Cinnamomum zeylanicum	Cinnamon
Citrus aurantium	Orange
Citrus grandis	Grapefruit
Citrus medica limonum	Lemon
Citrus medica limonum – Argentina	Lemon
Citrus paradisi	Grapefruit
Citrus sinensis	Orange
Citrus sinensis – Brazil	Orange
Citrus sinensis – Florida	Orange
Commiphora myrrha	Myrrh
Cupressus sempervirens	Cypress
Cymbopogon citrates	Lemongrass
Cymbopogon martini	Palmarosa
Cymbopogon nardus	Citronella

Table 2.1 continued The fity-four essential oils obtained from commercial suppliers for use in combination with *L. angustifolia*.

Scientific name	Common name
Daucus carota	Carrot Seed
Eucalyptus globulus	Eucalyptus
Eugenia caryophyllus	Clove
Foeniculum dulce	Fennel
Hyssopus officinalis	Hyssop
Juniperus communis (berries)	Juniper
Juniperus virginiana (China)	Cedarwood
Juniperus virginiana (Virginia)	Cedarwood
Laurus nobilis	Bay
Litsea cubeba	Litsea cubeba
Matricaria chamomiua	Chamomile
Melaleuca alternifolia	Tea-tree
Melaleuca viridiflora	Niaouli
Mentha piperita	Peppermint
Mentha piperita (America)	Peppermint
Mentha piperita (China)	Peppermint
Myrtus communis	Myrtle
Ocimum basilicum	Basil
Origanum majorana	Marjoram
Pelargonium odoratissimum	Geranium
Piper nigrum	Black Pepper
Pogostemon patchouli	Patchouli
Rosmarinus angustifolia	Rosemary
Rosmarinus officinalis	Rosemary
Salvia sclarea	Sage
Santalum album	Sandalwood
Styrax benzoin	Benzoin
Tagetes minuta	Tagetes
Thymus vulgaris	Thyme

The essential oils supplied were stored in amber vials and kept in the refridgerator (at a constant temperature of 4 °C) until their use in the antimicrobial assays.

2.2. Chemical analysis

The composition of each oil was determined by gas chromatography coupled to a mass spectrometer (GC-MS) to confirm purity and authenticity. The GC-MS (Agilent 6890 N GC system) was coupled directly to a 5973 MS equipped with a HP-Innowax polyethylene glycol column (60 m × 250 μm i.d. × 0.25 μm film thickness). A volume of 1 μL was injected (using a split ratio of 200:1) with an autosampler at 24.79 psi and an inlet temperature of 250 °C. The GC oven temperature was placed at 60 °C for 10 min, then 220 °C at a rate of 4 °C/min for 10 min and followed by a temperature of 240 °C at a rate of 1 °C/min. Helium was used as a carrier gas at a constant flow of 1.2 mL/min. Spectra was obtained on electron impact at 70 eV, scanning from 35 to 550 m/z. The percentage composition of the individual components were quantified by integration measurements using flame ionization detection (FID, 250 °C) and n-alkanes as reference points in the calculation of relative retention indices (RRI). Component identifications were made by comparing mass spectra from the total ion chromatogram (van Vuuren *et al*., 2010).

2.3. Major chemical constituents

The major chemical constituents of the most promising synergistic essential oil combinations were considered for analysis to determine the interactive role of these compounds. The major chemical constituents were selected according to the determined chemotype and combined in various combinations with major compounds from *L. angustifolia*. These major chemical constituents were stored in amber vials in a refridgerator until their use in the antimicrobial assays.

2.4. Conventional antimicrobial agents

The antimicrobial agents selected for analysis were prepared to a concentration of 0.1 mg/ml using sterile water or a 70% ethanol solution as a solvent in accordance to preparation suggestions made by the manufacturer, Sigma Aldrich®, South Africa. Chloramphenicol (≥ 98.0% purity, Sigma-Aldrich®), ciprofloxacin (≥ 98.0% purity, Sigma-Aldrich®), fusidic acid

(≥ 98.0% purity, Sigma-Aldrich®) and nystatin (70.0% purity, Sigma-Aldrich®) were selected for analysis due to their indication in respiratory and topical infection disease states.

2.5. Antimicrobial assays

2.5.1 Media and culture preparation

All media (Tryptone Soya agar, broth (Oxoid)) were prepared according to the instructions provided by the supplier, Sigma Aldrich® i.e. weighed, dissolved in distilled water and autoclaved (Butterworth) at 121°C for 15 min. After sterilization, all media was pre-incubated at a temperature of 37 °C for 24 hours to confirm sterility before further use.

The micro-organisms chosen for this study were selected to represent the infection states most commonly treated by essential oils. The essential oil *L. angustifolia* is most commonly used for the treatment of respiratory and topical infections (Dunning, 2006), thus, the following Gram-positive micro-organisms were selected; methicillin-resistant *Staphylococcus aureus* (ATCC 43300), a clinical strain of methicillin-resistant *Staphylococcus aureus* (ATCC 6437938), methicillin-gentamicin resistant *Staphylococcus aureus* (ATCC 33592), *Staphylococcus aureus* (ATCC 6538), a clinical strain of *Staphylococcus aureus* (ATCC 6437938), *Staphylococcus epidermidis* (ATCC 2223), a clinical strain of *Staphylococcus epidermidis* (BA16), vancomycin-resistant *Enterococcus faecalis* (ATCC 51299) and a clinical strain of *Enterococcus faecalis* (6059015). *Klebsiella pneumoniae* (ATCC 13883) and *Pseudomonas aeruginosa* (ATCC 27858) were selected to represent Gram-negative bacteria; while *Cryptococcus neoformans* (ATCC 11093), *Candida tropicalis* (ATCC 201380) and *Candida albicans* (ATCC 10231) were chosen to represent the yeasts.

In order to evaluate combined efficacies, three micro-organisms were selected; *Staphylococcus aureus* (ATCC 6538); *Pseudomonas aeruginosa*, (ATCC 27858) and *Candida albicans*, (ATCC 10231). All ATCC strains of micro-organism were obtained from Davies Diagnostics Pty Ltd (South Africa), while all clinical strains were received from Dr. M. Patel (Department of Oral Microbiology, The University of the Witwatersrand). The Clinical Laboratory Standards Institute (CLSI), formerly known as the National Committee for Clinical Laboratory Standards (NCCLS) (2003) guidelines were used to ensure that

accurate microbiological assay and transfer techniques were followed. Stock cultures were retained at –20 °C, subcultured onto Tryptone Soya agar, incubated at optimum temperatures and checked for purity. Isolated pure colonies were selected and transferred onto Tryptone Soya agar and thereafter kept viable by subculturing weekly for stock culture maintenance.

2.5.2. Minimum inhibitory concentration (MIC) micro-titre plate assay

The essential oils (starting concentration of 32 mg/ml)/major chemical constituents (starting concentration of 32 mg/ml)/conventional antimicrobial agents (starting concentration of 0.1 mg/ml) were evaluated for antimicrobial efficacy using the micro-dilution MIC assay (Eloff, 1998). The micro-titre plates were prepared by adding 100 µl of sterile, distilled water into each well of the 96-well micro-titre plate using aseptic technique. The essential oil/major chemical constituent/conventional antimicrobial agent was added to the micro-titre plate, at a volume of 100 µl when investigated independently and at a ratio of 1:1 (50: 50 µl) when investigated in combination. These were serially diluted to concentrations of 8, 4, 2, 1, 0.5, 0.25, 0.13 and 0.06 mg/ml (Figure 2.1). The micro-organisms for testing were diluted using sterile Tryptone Soya broth at a 1:100 dilution in order to achieve an approximate concentration of $1x10^6$ colony forming units (CFU) per ml. Cultures were added to all the wells of their respective micro-titre plates, at a volume of 100 µl. The micro-titre plates were then sealed with a sterile adhesive sealing film to prevent any essential oil loss due to evaporation when incubated. The micro-titre plates were incubated under optimal conditions of 37 ºC for 24 hours for bacteria and 37 ºC for 48 hours for yeasts. After incubation, 0.4 mg/ml of *p*-iodonitrotetrazolium violet (Sigma-Aldrich) solution (INT) was added into each well (40 µl). The microtitre plates inoculated with bacteria were examined after six hours to determine a colour change, while the yeasts were examined after 24 hours of INT addition. Viable micro-organisms interact with the INT solution to cause a colour change from clear to a red-purple colour. The lowest dilution with no colour change was considered as the MIC for that oil (Angeh, 2006). These tests were done in triplicate and further repetitions conducted where necessary to maintain accuracy.

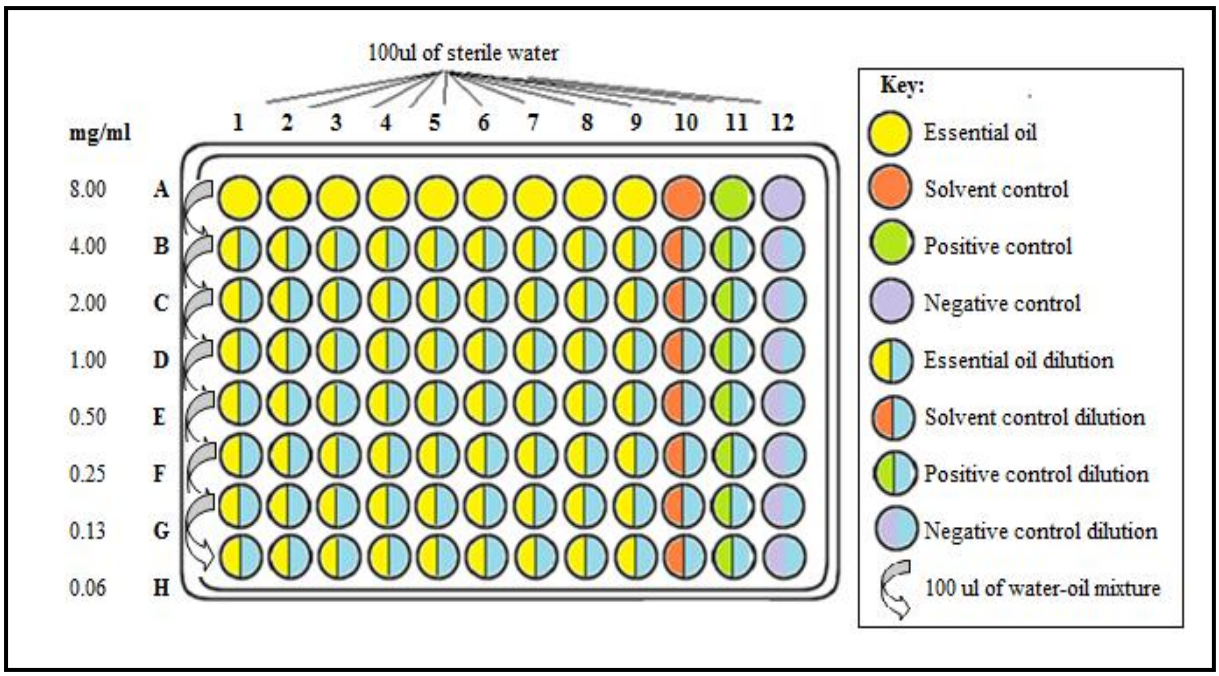


Figure 2.1 Serial dilutions of essential oils using a 96 well micro-titre plate.

2.5.2.1. Antimicrobial assay controls

Positive and negative controls were included in each assay. The positive control was 0.01 mg/ml ciprofloxacin for bacteria, and 0.10 mg/ml amphotericin B for yeasts. These conventional antimicrobial controls were included to ensure microbial susceptibility. MIC values obtained for the conventional antimicrobial agents investigated were compared to breakpoints given by the KnowledgeBase antimicrobial index. The acceptable control ranges for ciprofloxacin and amphotericin B against the pathogens used in the MIC assays are shown in Table 2.2. The acceptable control ranges for chloramphenicol, fusidic acid and nystatin against the micro-organisms tested are given in Table 2.3. Media and culture controls, such as Tryptone Soya broth (Oxoid) were included to confirm sterility and viability respectively. The controls were added to the micro-titre plate, at a volume of 100 µl and then serially diluted as in the methodology outlined in Section 2.5.2.

Table 2.2 Acceptable control ranges for MIC assays (ciprofloxacin for bacteria and amphotericin B for yeasts).

Micro-organism	Reference number	MIC control ranges (µg/ml)*
Methicillin-resistant *Staphylococcus aureus* (MRSA)	ATCC 43300	0.13 - 1.00
Methicillin-resistant *Staphylococcus aureus* - Clinical strain	6437938	0.12 - ≥ 128
Methicillin-gentamicin resistant *Staphylococcus aureus* (MGRSA)	ATCC 33592	0.12 - ≥ 128
Staphylococcus aureus	ATCC 6538	0.12 – 0.50
Staphylococcus aureus – Clinical strain	6437938	0.60 - 0.61
Staphylococcus epidermidis	ATCC 2223	0.30 - 2.50
Staphylococcus epidermidis – Clinical strain	BA 16	0.30 - 2.50
Vancomycin-resistant *Enterococcus faecalis*	ATCC 51299	0.25 - >16.00
Enterococcus faecalis – Clinical strain	6059015	5.00 - 20.00
Klebsiella pneumoniae	ATCC 13883	0.03 - 0.60
Pseudomonas aeruginosa	ATCC 27853	0.13 - 1.00
Cryptococcus neoformans	ATCC 11093	0.70
Candida tropicalis	ATCC 201380	1.25 - 2.50
Candida albicans	ATCC 10231	0.03 - 0.50

*MIC ranges determined by KnowledgeBase antimicrobial index (http://antibiotics.toku-e.com/) and NCCLS guidelines.

Table 2.3 Micro-organisms with acceptable control ranges as determined by KnowledgeBase antimicrobial index (http://antibiotics.toku-e.com/) for MIC assays (ciprofloxacin for bacteria and amphotericin B for yeasts).

Micro-organism	Reference number	MIC control ranges (µg/ml)*		
		Chloramphenicol	Fusidic acid	Nystatin
Candida albicans	ATCC 10231	<4.0*	NA	0.49 - 400*
Staphylococcus aureus	ATCC 6538	0.1 – 15.6*	0.1 - >32.0*	NA
Pseudomonas aeruginosa	ATCC 27853	0.062 - >32.0*	NA	NA

2.5.3. Combination studies

2.5.3.1. Fractional inhibitory concentration (FIC) of 1:1 combinations

For the 1:1 combinations, the fractional inhibitory concentration (FIC) and the FIC index (ΣFIC) was calculated to determine the interaction between *L. angustifolia* and a selection of essential oils/major chemical constituents/conventional antimicrobial agents. The FIC and the

ΣFIC were calculated by dividing the MIC value of the combination (taking into account half the concentration is attributed due to 1:1 mixes) with the MIC value of each essential oil/major chemical constituent/conventional antimicrobial agent placed in the combination. The ΣFIC was then calculated by adding these two FIC values together.

Equation 2.1:

$$\Sigma FIC = \frac{MIC^{A*} \text{ in combination}}{MIC^{A} \text{ alone}} + \frac{MIC^{B**} \text{ in combination}}{MIC^{B} \text{ alone}}$$

* A= *Lavandula angustifolia* essential oil or major chemical constituent A as determined by research undertaken.

**B= selection of essential oils as outlined in Appendix B/ major chemical constituent B as determined by research undertaken/conventional antimicrobial agent.

Previously, the ΣFIC for each combination was interpreted as synergistic where the ΣFIC was less than 1.00, and antagonistic with an ΣFIC of greater than 1.00. This model has been amended as studies have shown variations and reproducibility errors when using this limited scale (Odds, 2003). The ΣFIC for each combination was thus adapted and interpreted as synergistic where the ΣFIC was less than or equal to 0.50. For additive properties the ΣFIC was interpreted as greater than 0.50 but less than or equal to 1.00. For indifference, ΣFIC values are greater than 1.00 but less than or equal to 4.00 and antagonism occurs when an ΣFIC greater than 4.00 is observed (van Vuuren and Viljoen, 2011).

2.5.3.2. Variable ratio combinations

In order to determine what antimicrobial interactions could be apparent if variable concentrations of *L. angustifolia* and an essential oil (starting concentration of 32 mg/ml)/major chemical constituent (starting concentration of 32 mg/ml)/conventional antimicrobial agent (starting concentration of 0.1 mg/ml) were mixed, samples were selected and combined in nine ratios i.e. 9:1; 8:2; 7:3; 6:4; 5:5; 4:6; 3:7; 2:8 and 1:9. MIC values were determined for all nine ratios as well as for the independent essential oils/major chemical compounds/ conventional antimicrobial agents. These MIC values were then used to calculate the interaction by examining the data for each ratio in relation to the MICs for the oil/major

chemical constituent/conventional antimicrobial agent independently. Isobolograms were constructed using mean MIC values of the combinations as ratios on GraphPad Prism®, version five software (Suliman *et al*., 2010). All points below or on the 0.5: 0.5 line (green quadrant) on the isobologram were interpreted as synergistic (Figure 2.2). Points between the 0.5: 0.5 and 1.0: 1.0 line (blue quadrant) were interpreted as additive and points above the 1.0: 1.0 line and including the 4.0 line were observed as non-interactive (yellow quadrant). Points above the 4.0: 4.0 line (purple quadrant) were considered antagonistic (van Vuuren and Viljoen, 2011).

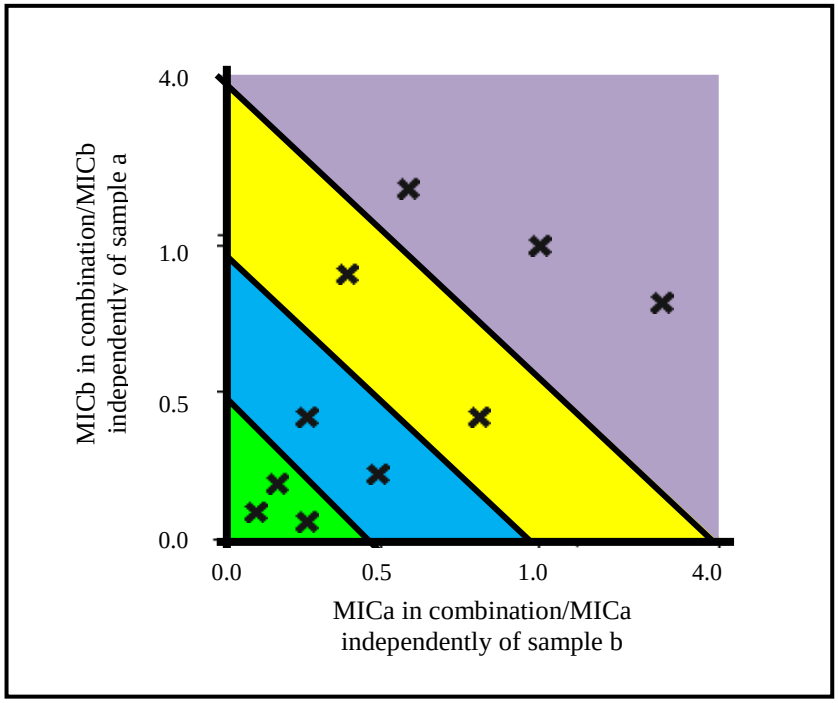


Figure 2.2 A diagrammatic explanation to the interpretation of isobolograms (van Vuuren and Viljoen, 2011). Where 'a' represents *L. angustifolia*/major chemical constituent A and 'b' represents one of the other (Appendix C) oils/major chemical constituent B/conventional antimicrobial agent.

2.6. Data analysis of triple essential oil combinations

The Design of Experiments (MODDE 9.1®) software was used to identify and optimize the most promising combinations for synergy based on the antimicrobial activity of *Lavandula angustifolia* and its combinations in various ratio mixes. Design of Experiments (MODDE 9.1® Umetrics AB, Umea, Sweden) aids in identifying optimal synergistic interactions

between three samples, in this case essential oils. This is performed by comparing the various combinations against the data obtained from the individual MIC values of the essential oils under investigation (Eriksson *et al*., 2008).

2.6.1. Preparation of media and microbial cultures

Culture and media preparation were undertaken according to the CLSI (2003) guidelines as outlined in Section 2.5.1. The micro-organism selected for analysis was *C. albicans* (ATCC 10231) as the literature specified the use of the triple combinations selected against this micro-organism.

2.6.2. Data software preparation

Once the essential oil combinations had been identified (starting concentration of 32 mg/ml) and the micro-organism selected for analysis, a worksheet was formulated using MODDE® software (Table 2.4).

Table 2.4 Worksheet generated by MODDE® for the analysis of essential oil triple combinations.

Exp No	Run Order	Volume of essential oil (µl)			MIC (mg/ml)
		Essential oil 1	Essential oil 2	Essential oil 3	
1	22	0	0	0	To be inserted once experiment has been completed
2	23	100.0	0	0	
3	10	0	100.0	0	
4	13	50.0	50.0	0	
5	9	0	0	100.0	
6	24	50.0	0	50.0	
7	12	0	50.0	50.0	
8	18	33.3	33.3	33.3	
9	21	33.3	33.3	33.3	
10	17	33.3	33.3	33.3	
11	16	33.3	33.3	33.3	
12	6	0	0	0	

Table 2.4 continued Worksheet generated by MODDE® for the analysis of essential oil triple combinations.

Exp No	Run Order	Volume of essential oil (µl)			MIC (mg/ml)
		Essential oil 1	Essential oil 2	Essential oil 3	
13	1	100.0	0	0	To be inserted once experiment has been completed
14	3	0	100.0	0	
15	5	50.0	50.0	0	
16	2	0	0	100.0	
17	14	50.0	0	50.0	
18	4	0	50.0	50.0	
19	15	33.3	33.3	33.3	
20	7	33.3	33.3	33.3	
21	8	33.3	33.3	33.3	
22	19	33.3	33.3	33.3	

This worksheet is created by MODDE® in which the ratios in which the essential oils should be combined and the run order is specified. The ratios selected for combination by MODDE® allows for the software to generate the optimal concentration ratio for the greatest antimicrobial effect, while the specification in the run order helps to limit possible operator bias for the outcome of MIC values per ratio combination. This worksheet is used to carry out the MIC assay of the essential oil combination. These tests were done in duplicate to maintain accuracy.

2.6.3. Minimum inhibitory concentration micro-titre plate assay

2.6.3.1. Initial screening of essential oil combinations

The MIC were determined using the micro-dilution method (Eloff, 1998) according to the run order specified by the worksheet created in Section 2.6.2. However, variance in methodology occurred when compared to previous methods undertaken as the MIC values afforded to each well of the micro-titre plate were refined to narrow the increments. This was achieved by altering the dilution factor from 0.50 to 0.75. This is done in order to reduce the error commonly encountered with the doubling dilution methodology of MIC analysis (van Vuuren, 2008). Therefore, initially the essential oils identified for analysis (*L. angustifolia*, *C. atlantica, C. sinensis*, *D. carota* and *T. vulgaris*) were diluted to a starting concentration of

32 mg/ml using acetone as the solvent. The concentration of the essential oil was diluted by removing 100 µl of the essential oil and diluting in 300 µl of sterile water. This dilution of the essential oil continued until eight dilutions (A-H) had been achieved (Figure 2.3).

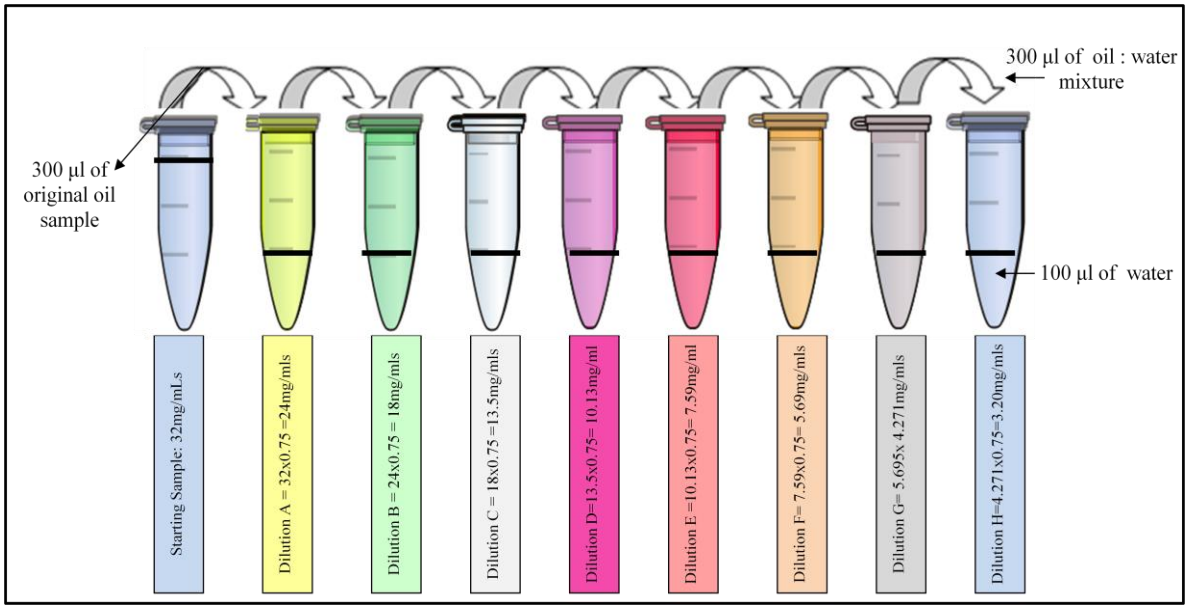


Figure 2.3 Dilution of essential oil in order to achieve narrow MIC increments.

The essential oil concentration per eppendorf was then calculated according to the following equation:

Equation 2.2:

Concentration of essential oil B = Concentration of essential oil A x dilution factor

Once each essential oil in the triple combination had been diluted as in Figure 2.3, the essential oil dilutions in the eppendorfs were then added to the micro-titre plate, according to the volume specified in the worksheet (Table 2.4) for assaying (Figure 2.4)

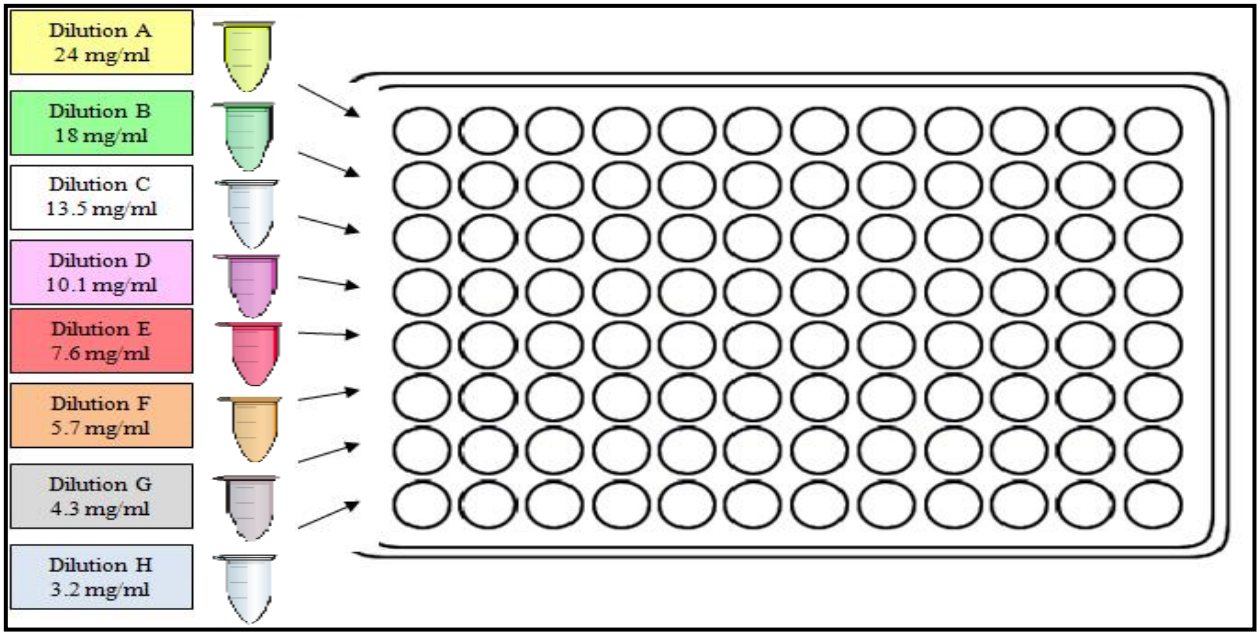


Figure 2.4 Diluted essential oil added to representative column of micro-titre plate according to volume specified in Table 2.4.

Culture was then added to all the wells of the micro-titre plates, at a volume of 100 µl. This caused the concentration of the essential oil to be diluted further due to the addition of the liquid culture to the well. The MIC values attributed to each well of the micro-titre plate are illustrated in Figure 2.5.

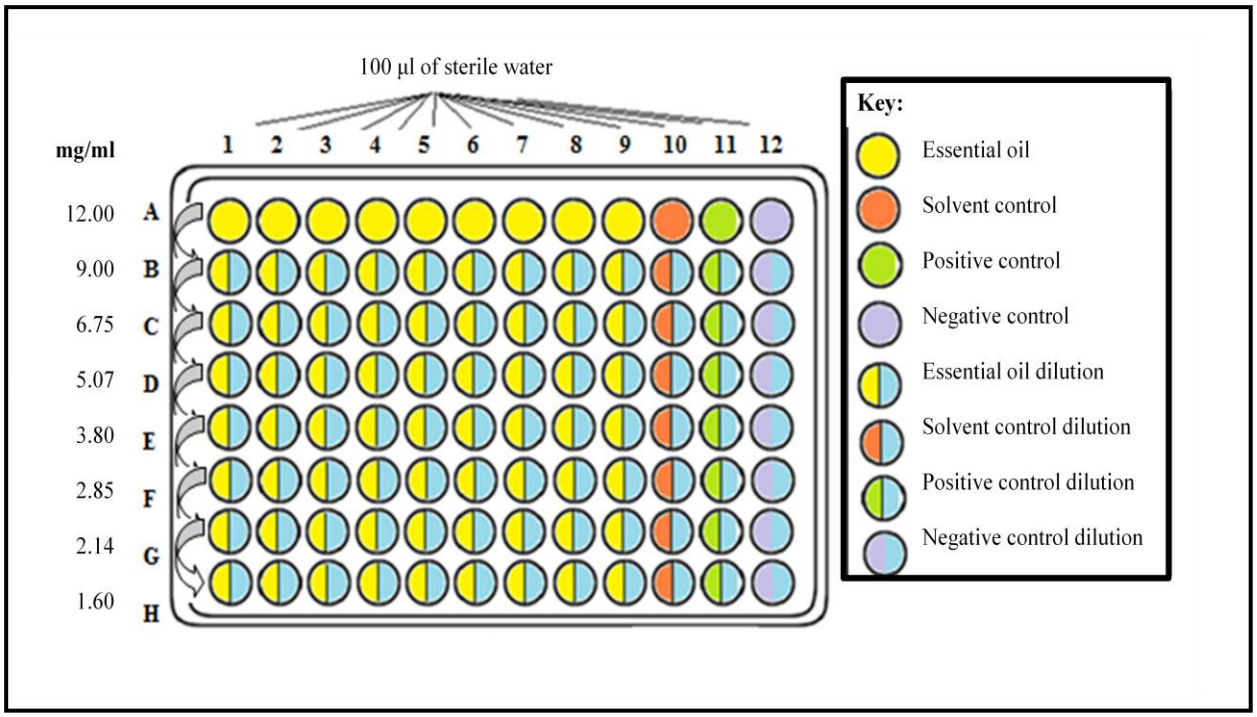


Figure 2.5 MIC values attributed to each well of the micro-titre plate.

The micro-titre plates were then sealed with a sterile adhesive sealing film to prevent any essential oil loss due to evaporation when incubated. The micro-titre plates were incubated under optimal conditions of 37 ºC for 24 hours for bacteria and 37 ºC for 48 hours for yeasts. After incubation, 0.4 mg/ml of *p*-iodonitrotetrazolium violet solution (INT) was added into each well (40 µl) of the micro-titre plates upon which colour changes were noted after eight hours of addition, and the MIC values determined. Positive and negative controls were prepared and included in each assay according to the method outlined in Section 2.5.2.1. The acceptable control ranges for ciprofloxacin and amphotericin B against the pathogen used in the MIC assays are shown in Table 2.1.

2.6.4. Data analysis of essential oil combinations

Once the data of the various combination MIC values was inserted into the system, the software identified graphically, strong interactions through the production of a series of graphs. These graphs included the replicate plot, histogram, summary of fit plot, co-efficient plot, residual N-plot, observed versus predicted plot and the response contour plot. Once these parameters have indicated the software's ability to predict future MIC results of the combination, the optimizer function is used. The optimizer determines what ratio combinations the essential oils should be placed in order to obtain the best possible MIC value for the combination.

2.6.4.1. Replicate plot

The replicate plot displays the measured MIC values for each experimental run (Figure 2.6). Replicates of the same experiment are plotted on the same stick, with run numbers overlapping. The ideal outcome is to have little or no variation occur between replicates of the same experiment (ie. to have the numbers of the replicates of each experiment fall on top of one another). This graph indicates the reproducibility of the experiment.

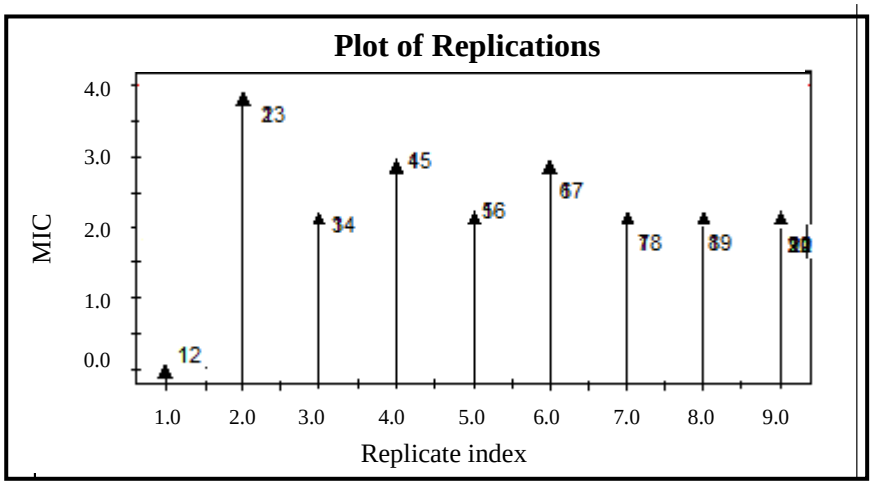


Figure 2.6 The plot of replications.

2.6.4.2. Histogram

The histogram plot displays the distribution of MIC results (Figure 2.7). It is important to obtain an evenly distributed histogram in the form of a bell curve distribution for further analysis, as the Design of Experiments (MODDE®) software uses statistics based on linear models. If the histogram is not of even distribution (i.e. not bell shaped curve) it can undergo transformation. This transformation causes the MIC results obtained to be "transformed" into a linear model for further analysis.

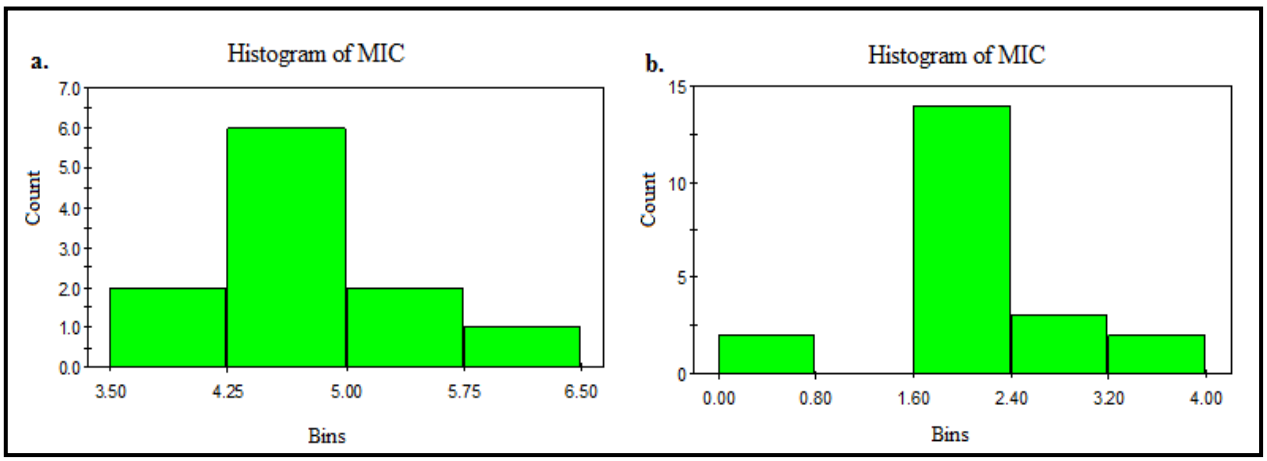


Figure 2.7 Histogram of normal distribution (a) and histogram requiring transformation (b).

2.6.4.3. Summary of fit plot

The summary of fit plot displays four important performance indicators, namely the R^2, Q^2, Model Validity and Model Reproducibility (Figure 2.8).

R^2 is indicated on the graph as the green bar and is known as the indicator for "goodness of fit". R^2 indicates the software's ability to explain and predict future error in experiments. A strong ability to which the software is able to predict future error and as such create trustworthy predictions of future MIC values typically presents with an R^2 value of ≥ 0.50.

Q^2 is indicated on the graph as the dark blue bar and is the indicator for the models ability to predict future results based on the results obtained during screening (Table 2.4), and as such is also known as the "goodness of prediction". A Q^2 value of 0.1 to 0.50 is necessary for the software to predict future outcomes.

Model validity is indicated on the graph as the yellow bar and indicates how trustworthy future predictions made by the software are. A low model validity (< 0.25) shows that future predictions should not be trusted however, a model validity of zero can occur for experiments which show no possible error (i.e. zero pure error). Experiments with zero pure error shows no model validity on the summary of fit plot, but the future predictions made by the software are considered trustworthy.

Model reproducibility is indicated on the graph as the light blue bar and indicates the ease to which the experiments can be reproduced. An experiment is considered valid if the model reproducibility is ≥ 0.50.

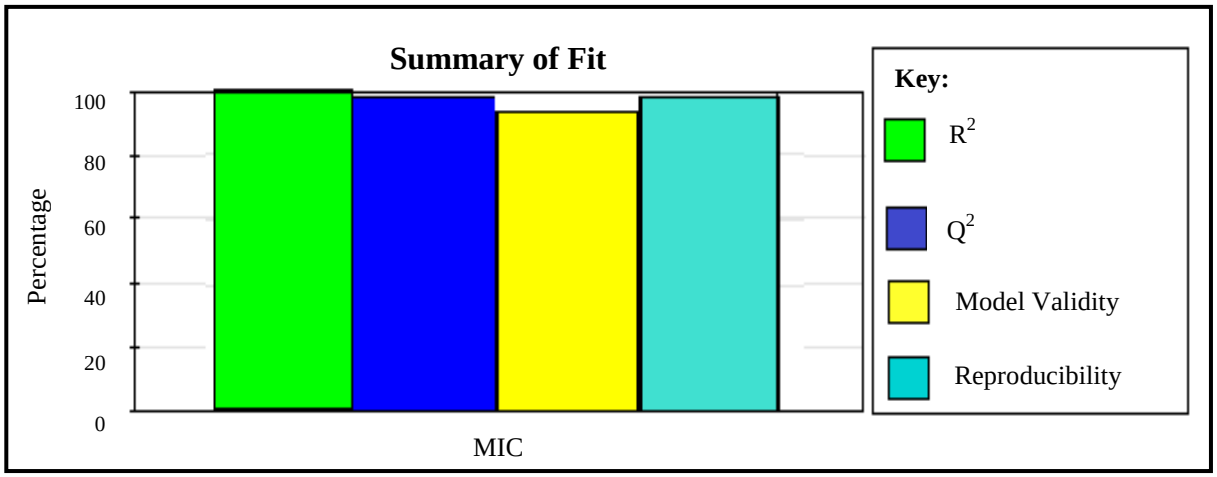


Figure 2.8 The summary of fit plot.

2.6.4.4. Co-efficient plot

The co-efficient plot graphically displays the impact of each essential oil on the MIC outcome of the combination (Figure 2.9).

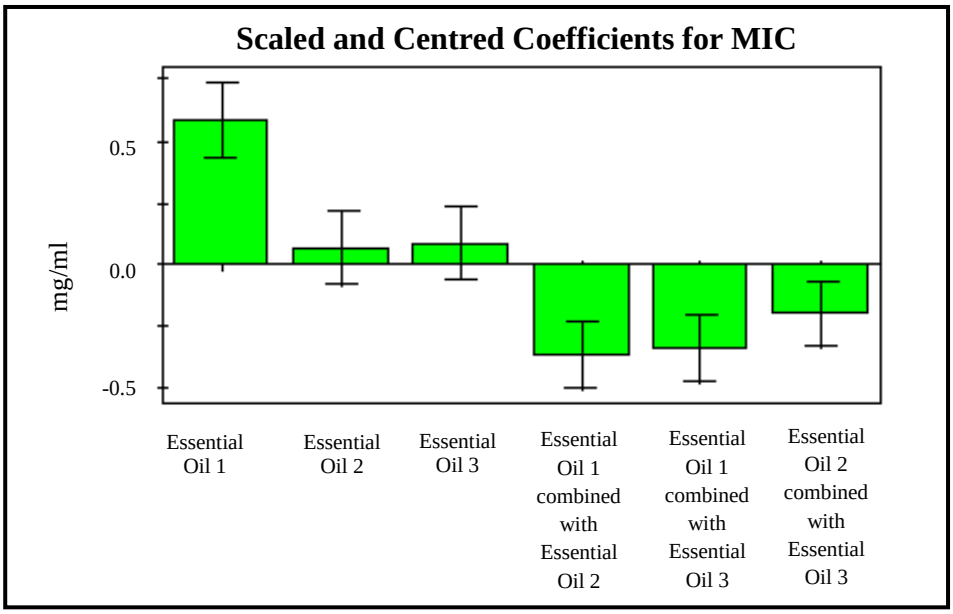


Figure 2.9 The co-efficient plot.

Typically, a significant essential oil in the combination is tall with a large confidence variable (indicated by the T-line), such is in the case of essential oil 1 in Figure 2.9. While essential oils in the combination that present with short depths or confidence variables that cross the 0

line are considered insignificant to the outcome of the MIC of the combination, such as essential oil 2 and essential oil 3 in Figure 2.9. When interpreting the co-efficient plot for MIC analysis, variables that plot closer to zero and into the negative region are considered to be more significant than those found in the positive region of the graph (i.e. The interaction between essential oil 1 and essential oil 2 is more important to the outcome of the combination MIC than the interaction between essential oil 2 and essential oil 3). This interpretation is based on the antimicrobial guidelines that the lower the MIC the greater the antimicrobial outcome.

2.6.4.5. Residual N-plot

The residual N-plot identifies the probability of noise (error) in a data set (Figure 2.10). This allows the researcher to determine if the results should be trusted or removed, as random error causes the software prediction ability to be affected. Values that plot along the diagonal line are considered to be trustworthy. Some values do not plot along the expected line, but are not considered untrustworthy, rather only a point where the MIC value cannot be predicted by the interactions between essential oils.

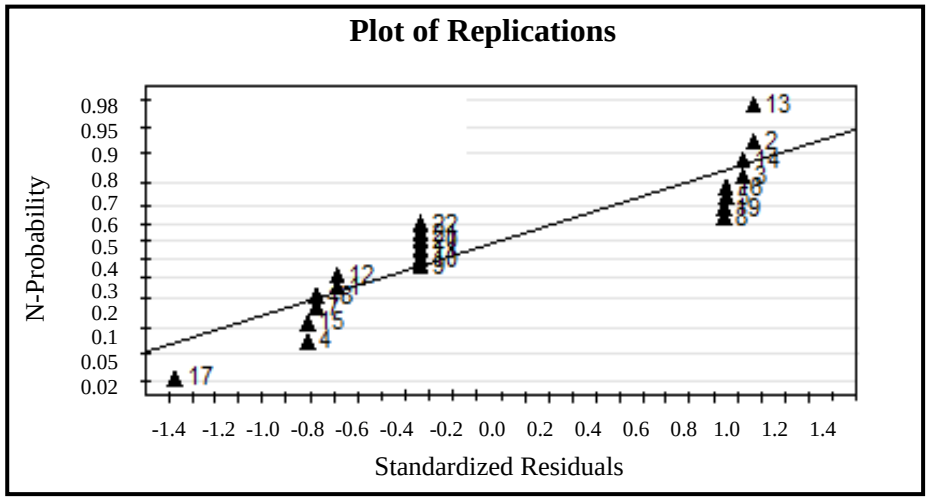


Figure 2.10 The residual N-plot.

2.6.4.6. Observed versus predicted plot

The observed versus predicted plot (Figure 2.11) identifies the efficacy of the software to predict future results based on data obtained during screening (Table 2.4). Points closest to the regression line and closer to each other indicate high predictability of the software. Some values do not plot along the expected line, but are not considered untrustworthy, but rather a point where the MIC value cannot be predicted by the interactions between essential oils.

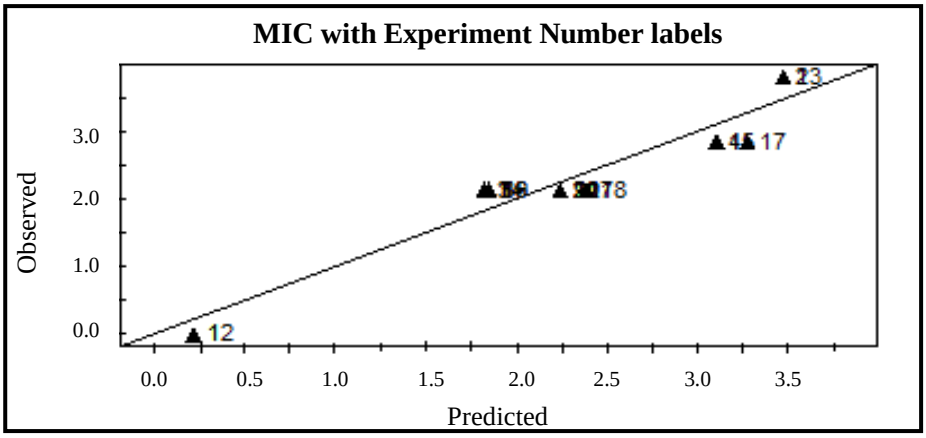


Figure 2.11 The observed versus predicted plot.

2.6.4.7. Response contour plot

The response contour plot allows for the identification of ratios of the combinations that demonstrate the best and worst overall antimicrobial effect (Figure 2.12). Essential oil combinations in the red region are shown to produce high MIC values, while essential oil combinations in the green region demonstrate optimal, lower MIC values. In the green region predicted MIC values are given with ratio mixes of the essential oils in the combination and are expected to give the predicted MIC response. From the response contour plot a visual account for the best essential oil blend is given.

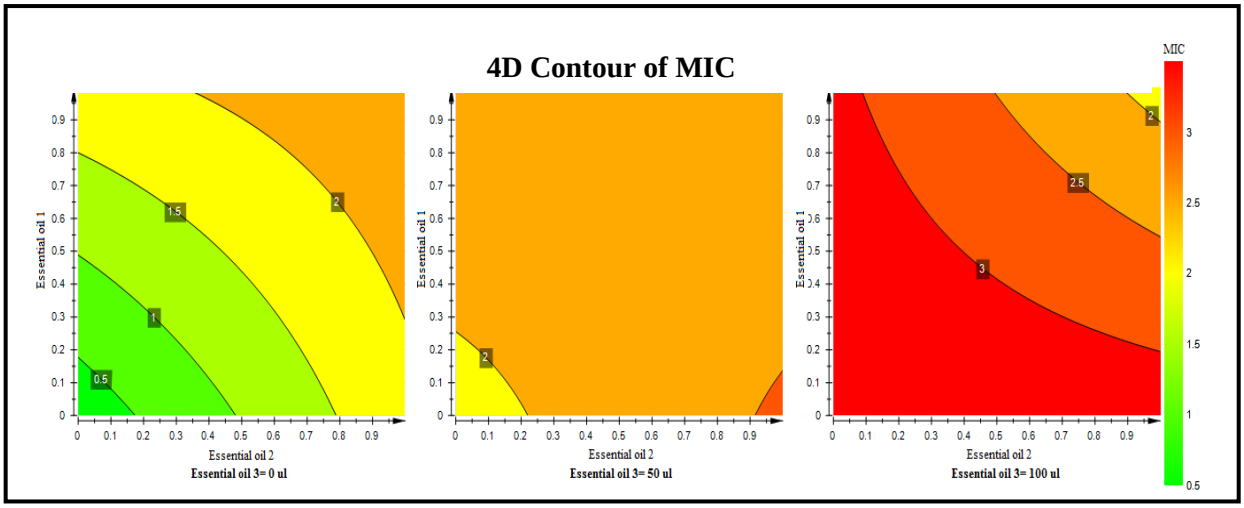


Figure 2.12 The response contour plot.

CHAPTER 3
IDENTIFICATION OF ESSENTIAL OIL CONSTITUENTS

3.1. Introduction

3.1.1. Essential oil chemistry

The chemistry of essential oils is a complex and diverse subject, as there are many variations of chemical compounds in essential oils. Identifying and understanding the chemical composition of essential oils under analysis is an important practice as essential oil composition changes under certain conditions. *Lavandula angustifolia* essential oil is considered high in quality, if the oil contains a high proportion of esters such as linalyl acetate (Department of Agriculture, Forestry and Fisheries, 2009). The concentration of these esters drop significantly in cold weather, whereas in warmer weather essential oil is lost due to evaporation (Department of Agriculture, Forestry and Fisheries, 2009). Thus, in South Africa *L. angustifolia* is harvested during the end of December and early January due to the warmer weather conditions (Department of Agriculture, Forestry and Fisheries, 2009). The essential oil of *Thymus vulgaris* is just as easily affected by environmental conditions.

T. vulgaris essential oil obtained from plants grown at the bottom of a mountain are more likely to contain the chemical compound, geraniol (Clarke, 2008). *T. vulgaris* essential oil isolated from plants grown in the middle of the mountain contain higher levels of monoterpene phenols, while *T. vulgaris* essential oil obtained from plants grown at the peak of a mountain are more likely to contain a large amount of linalool (Clarke, 2008). These variances may result in different antimicrobial outcomes when assayed. This phenomenon of diversity among essential oils of the same name and species is a common occurrence and has been extensively reported (Cosentino *et al*., 1999; Pitarokili *et al*., 2002; Skaltsa *et al*., 2003; Jirovetz *et al*., 2006; Pawar and Thaker, 2006; Mayaud *et al*., 2008; O'Bryan *et al*., 2008; Chao *et al*., 2008; Iten *et al*., 2009; Maxia *et al*., 2009; Hassiotis, 2010; Al-Hussaini and Mahasneh, 2011; Goren *et al*., 2011; Jayaprakasha and Rao, 2011). Thus when analysing *L. angustifolia* and other essential oils of importance in this study, it is necessary to record the chemistry of the essential oil under investigation.

The aim of this chapter is to identify the compounds in the essential oils selected for investigation. The analytical method used in this study to determine the composition of the selected essential oils is the use of gas chromatography coupled to mass spectroscopy (GC-MS). The combination of these two analytical methods allows for a more accurate determination of the compounds in the essential oil.

3.2. Results and Discussion

3.2.1. Essential oil chemistry

A chemical analysis of the essential oils used in this study was undertaken to determine the purity and specific chemotype of essential oil used in the present study. Peaks of percentage area ≥10% were considered to be major chemical constituents (van Vuuren and Viljoen, 2008). The major chemical constituents for each essential oil selected for analysis were identified and are given in Table 3.1 together with the confirmation of chemotype as found in literature.

Table 3.1 The chemical composition of the essential oils under investigation (for the sake of brevity only biomarkers and/or major compounds for each oil have been included).

Essential oil	Major constituent/s	Percentage abundance	Constituent class	Previous research conducted
Abies balsamea	*β*-Pinene	31.0	Monoterpene	Ross *et al.*,1996; Pichette *et al.*,2006
	Bornyl acetate	14.9	Monoterpene	
	δ-3-Carene	14.2	Monoterpene	
Andropogon muricatus	Zizanol	12.6	Sesquiterpene	Jain *et al*., 1982
Angelica archangelica (root)	*α*-Phellandrene	18.5	Monoterpene	Taskinen and Nykänen, 1979
	α-Pinene	13.7	Monoterpene	
Angelica archangelica (root)	*β*-Phellandrene	12.6	Monoterpene	Nykänen *et al*., 1991; Doneanu and Anitescu, 1998
	δ-3-Carene	12.1	Monoterpene	
Angelica archangelica (seed)	*β*-Phellandrene	59.2	Monoterpene	Nivinskienë *et al*., 2005
Anthemis nobilis	2-Methylbutyl-2-Methyl propanoic acid	31.5	Monoterpene	Camboulises, 1871
	Limonene	18.3	Monoterpene	
	3-Methylpentyl-2-Butenoic acid	16.7	Monoterpene	
	Isobutyl isobuterate	10.0	Monoterpene	
Artemisia dracunculus	Estragole	82.6	Monoterpene	Sayyah *et al*., 2004

Table 3.1 continued The chemical composition of the essential oils under investigation

Essential oil	Major constituent/s	Percentage abundance	Constituent class	Previous research conducted
Canarium luzonicum	Limonene	47.1	Monoterpene	Villanueva *et al*., 1993
	Elemol	17.8	Sesquiterpene	
	α-Phellandrene	12.5	Monoterpene	
Cananga odorata (heads)	Benzyl acetate	31.9	Monoterpene	Oyen and Dung, 1999
	Linalool	27.0	Monoterpene	
	Methyl benzoate	10.4	Monoterpene	
Cananga odorata	Bicyclosesquiphellandrene	19.5	Sesquiterpene	
	β-Farnesene	13.9	Sesquiterpene	
Carum carvi	Limonene	27.6	Monoterpene	Lacobellise *et al.*,2005
	Carvone	67.5	Monoterpene	
Cedrus atlantica	*α*-Cedrene	27.1	Sesquiterpene	Adams, 1987; Eller *et al.*, 2010
	Cedrol	21.7	Sesquiterpene	
	Thujopsene	17.1	Sesquiterpene	
Cinnamomum zeylanicum	Eugenol	80.0	Monoterpene	Krishna *et al.*,1946; Mallavarapu *et al.*, 1995
Citrus aurantium	Linalyl acetate	54.9	Monoterpene	Gurib-Fakim and Demarne, 1995
	Linalool	21.1	Monoterpene	
Citrus grandis	Limonene	74.8	Monoterpene	Njorogne *et al.*,2005
Citrus medica limonum	Limonene	95.7	Monoterpene	Ibrahim *et al.*,2001
Citrus medica limonum – Argentina	Limonene	68.9	Monoterpene	
Citrus paradisi	Limonene	95.2	Monoterpene	Njorogne *et al.*,2005; Khan *et al*.,2007; Hosni *et al*., 2010
Citrus sinensis	Limonene	93.2	Monoterpene	Hosni *et al*., 2010
Citrus sinensis – Brazil	Limonene	95.3	Monoterpene	Michaelakis *et al.*,2009; Hosni *et al*., 2010
Citrus sinensis – Florida	Limonene	94.6	Monoterpene	
Commiphora myrrha	Furanoeudesma-1.3-diene	57.7	Sesquiterpene	Hanùs *et al.*,2005; de Rapper *et al.*,2012
	Lindastrene	16.3	Class unknown	
Cupressus sempervirens	*α*-Pinene	41.2	Monoterpene	Mazari *et al.*, 2010
	δ-3-Carene	23.7	Monoterpene	
Cymbopogon citrates	Geranial	44.8	Monoterpene	Onawunmi *et al*.,1984; Kasali *et al*.,2003; Bassolé, 2011

Table 3.1 continued The chemical composition of the essential oils under investigation

Essential oil	Major constituent/s	Percentage abundance	Constituent class	Previous research conducted
Cymbopogon martini	Geranial	81.1	Monoterpene	Raina *et al.*,2003; Nirmal *et al*.,2007; Padalia *et al.*,2011
Cymbopogon nardus	Citronellal	38.3	Monoterpene	de Billerbeck *et al*., 2001
	Geraniol	20.7	Monoterpene	
	Citronellol	18.8	Monoterpene	
Daucus carota	Carotol	44.4	Sesquiterpene	Jasicka-Misiak *et al.*,2004; Maxia *et al*.,2009
	β-Caryophyllene	5.7	Sesquiterpene	
	β-Bisabolene	5.3	Sesquiterpene	
Eucalyptus globulus	1,8-Cineole	58.0	Monoterpene	Dagne *et al*.,2000; Cimanga *et al.*,2002
	α-Terpineol	13.2	Monoterpene	
Eugenia caryophyllus	Eugenol	82.2	Monoterpene	Jirovetz *et al.*,2006
	Eugenol acetate	13.2	Monoterpene	
Foeniculum dulce	*E*-Anethole	79.1	Monoterpene	Chowdhury *et al.*,2009; Shahat *et al.*,2011
Hyssopus officinalis	Isopinocamphone	48.7	Monoterpene	Jankovsky and Lanica, 2002; Kizil *et al*., 2010
	Pinocamphone	15.5	Monoterpene	
Juniperus communis (berries)	*α*-Pinene	20.5	Monoterpene	Filipowicz *et al.*,2003; Wei and Shimbamoto, 2007
	Myrcene	13.7	Monoterpene	
	Bicyclosesquiphellandrene	10.7	Sesquiterpene	
Juniperus virginiana (China)	Thujopsene	29.8	Sesquiterpene	Adams, 1987; Eller *et al.*, 2010
	Cedrol	14.9	Sesquiterpene	
	α-Cedrene	12.4	Sesquiterpene	
Juniperus virginiana (Virginia)	Thujopsene	29.8	Sesquiterpene	Adams, 1987; Eller *et al.*, 2010
	Cedrol	15.0	Sesquiterpene	
	α-Cedrene	12.5	Sesquiterpene	
	β-Funebrene	6.4	Sesquiterpene	
Laurus nobilis	Eugenol	57.2	Monoterpene	Marzouki *et al*.,2009
	Myrcene	14.3	Monoterpene	
	Chavicol	12.7	Monoterpene	
Lavandula angustifolia	Linalyl acetate	36.7	Monoterpene	Roller, 2009; Soković *et al.*,2010
	Linalool	31.4	Monoterpene	
	Terpinen-4-ol	14.9	Monoterpene	
Litsea cubeba	Geranial	45.6	Monoterpene	Liu and Yang, 2012
	Nerol	31.2	Monoterpene	

Table 3.1 continued The chemical composition of the essential oils under investigation

Essential oil	Major constituent/s	Percentage abundance	Constituent class	Previous research conducted
Matricaria chamomiua	Bisabolene oxide A	46.9	Sesquiterpene	Matos *et al.,* 1993; Ganzera *et al.,* 2006
	β-Farnesene	19.2	Sesquiterpene	
Melaleuca alternifolia	Terpinen-4-ol	49.3	Monoterpene	Budhiraja *et al.*,1999; Hart *et al.*,2000; Calcabrini *et al.*,2004
	γ-Terpinene	16.9	Monoterpene	
Melaleuca viridiflora	1,8-Cineole	45.9	Monoterpene	Chebli *et al*., 2004
	α-Terpinene	21.0	Monoterpene	
Mentha piperita	Menthol	47.5	Monoterpene	Ansari *et al.*,2000; Bakkali *et al.*,2008
	Menthone	18.6	Monoterpene	
Mentha piperita (America)	Menthol	48.2	Monoterpene	
	Menthone	17.6	Monoterpene	
Mentha piperita (China)	Menthol	40.9	Monoterpene	
	Menthone	21.6	Monoterpene	
Myrtus communis	Myrtenyl acetate	28.2	Monoterpene	Mimica-Dukić *et al.*,2010; Brada *et al*.,2012
	1,8-Cineole	25.6	Monoterpene	
	α-Pinene	12.5	Monoterpene	
Ocimum basilicum	Linalool	54.1	Monoterpenol	Zhelijazkov *et al.,* 2008
Origanum majorana	1,8-Cineole	46.0	Monoterpene	Charai *et al*., 1996; Vági *et al*., 2005
	Linalool	26.1	Monoterpene	
Pelargonium odoratissimum	Citronellol	34.2	Monoterpene	Rana *et al.*,2002; Verma *et al*.,2010
	Geraniol	15.7	Monoterpene	
Piper nigrum	*β*-Caryophyllene	33.8	Sesquiterpene	Menon *et al.*,2003; Sasidharan and Menon, 2003
	Limonene	16.4	Monoterpene	
Pogostemon patchouli	Patchouli alcohol	37.3	Sesquiterpene	Wei and Shimbamoto, 2007
	α-Bulnesene	14.6	Sesquiterpene	
	α-Guaiene	12.5	Sesquiterpene	
Rosmarinus angustifolia	1,8-Cineole	48.0	Monoterpene	Kadri *et al.*,2011
Rosmarinus officinalis	Camphor	21.1	Monoterpene	Ait-Ouazzou *et al.*,2011; Soliman *et al*., 2011
	Verbenone	14.6	Monoterpene	
	Bornyl acetate	13.1	Monoterpene	
Salvia sclarea	Linalyl acetate	72.9	Monoterpene	Torres *et al.*,1997; Pitarokili *et al.,* 2002
	Linalool	11.9	Monoterpene	
Santalum album	*α*-Santalol	32.1	Sesquiterpene	Okugawa *et al.*,1995
Styrax benzoin	Cinnamyl alcohol	44.8	Monoterpene	Fernandez *et al.*,2003
	Benzene propanol	21.7	Monoterpene	

Table 3.1 continued The chemical composition of the essential oils under investigation

Essential oil	Major constituent/s	Percentage abundance	Constituent class	Previous research conducted
Tagetes minuta	(E)-*β*-Ocimene	41.3	Monoterpene	Chamorro *et al*.,2008
	E-Tagetone	11.2	Monoterpene	
	Verbenone	10.9	Monoterpene	
Thymus vulgaris	*p*-Cymene	39.9	Monoterpene	Bouhdid *et al.*,2006; Grosso *et al.*,2010; González *et al*., 2011
	Thymol	20.7	Monoterpene	
	γ-Terpinene	8.3	Monoterpene	

According to literature, variations in essential oil occur due to a number of external factors. These factors include climate, the season in which the plant is harvested, the part and maturity of the plant harvested, as well as soil mineralization and light intensity (Putievsky *et al*., 1986; Grella and Picci, 1988; Máthé *et al*., 1992; Länger *et al*., 1993; Piccaglia and Marotti, 1993; Li *et al*., 1996; Perry *et al*., 1997, Başer and Buchbauer, 2010).

The essential oil of *Angelica archangelica* was investigated in this study and the antimicrobial activity of the species determined for different parts of the plant. The major chemical constituents of *A. archangelica* root were determined as *α*-phellandrene (18.5%) and *α*-pinene (13.7%), while *β*-phellandrene (59.2%) was identified as the major chemical constituent of the essential oil obtained from the seed (Table 3.1). This result is confirmed by a study conducted by Pasqua *et al*. (2003) in which the chemical composition of the root oil of *A. archangelica* was determined by means of GC-MS analysis. From the study it was determined that *A. archangelica* root oil was comprised mainly of *α*-pinene with a percentage composition ranging from 23.9% to 32.7%. Another study conducted by Holm *et al.* (1997) further augments these findings in that the essential oil of *A. archangelica* root is comprised predominantly of *α*-pinene (18.9% to 42.4%), while the essential oil of *A. archangelica* seed comprised of 66.4% to 82.1% *β*-phellandrene. In a study conducted by Santos-Gomes and Fernandes-Ferreira (2001), variances in essential oil chemistry were determined between the leaves, stems and flowers of *Salvia officinalis* grown in Arouca, Portugal. The major constituents identified for the leaves included *α*-thujone (25.5%) and camphor (19.5%); while for the flowers *α* -thujone (17.7%), 1, 8-cineole (17.3%) and *β*-pinene (17.0%) were identified. For the essential oil obtained from the stem, *α*-thujone (55.1%) was determined as the only major chemical constituent. Another study conducted by Mirjalili *et al*. (2006) determined variances in essential oil chemistry between the flower and the fruit of *S.*

officinalis. The flower of *S. officinalis* comprised predominantly of 1, 8-cineole (22.3%) while the ripened fruit contained *α*-thujone (25.1%).

Another factor deemed responsible for differences in essential oil chemotype is the location at which the plant the essential oil was produced. A selection of essential oils from this study including *Citrus medica limonum, Citrus sinensis* and *Mentha piperita* were obtained from varying regions of the world and their chemotype investigated. Although the essential oils of the same species demonstrated the same chemical constituents, discrepancies occurred in the percentage of the constituent in the essential oils. The essential oils of *C. sinensis* obtained from Brazil and Florida were both identified as having limonene as a major chemical constituent; however the essential oil originating from Brazil was shown to be greater in this constituent (95.3%) than that of the essential oil obtained from Florida (94.6%) (Table 3.1). This variation is minimal (i.e. less than 10%) and as such is deemed as showing no variation. This minimal amount in variation was also noted for the essential oils of *M. piperita* while for the oil of *C. medica limonum,* a greater percentage difference in the abundance of the major chemical constituent was noted when comparing the same species. Both essential oils were identified as having limonene as a major chemical constituent however; the essential oil from Argentina was determined as having a significantly lower percentage (68.9%) of the compound comprising it when compared to that of the essential oil with unknown origin (95.7%).

In a study conducted by Milos *et al*. (2001), differences in chemical composition were identified for samples of *Satureja montana* and *Satureja cuneifolia* obtained from three different locations in Dalmatia, Croatia. For the sample of *S. montana* it was identified that the major chemical constituent of the species harvested in Biokovo was linalool (24.8%), while thymol was the major chemical constituent isolated from samples originating from Brač (11.0%) and Kozjak (20.6%). For the plant sample *S. cuneifolia,* distinct variances in essential oil chemistry were identified. The major chemical constituent for this sample originating from Biokovo was *β*-cubebene (11.1%), while *α*-pinene (10.9%) and linalool (18.2%) were the major constituents determined for the samples harvested from Brač and Kozjak, respectively. Another study conducted by Lis-Balchin *et al.* (1998) aimed to identify differences in essential oil composition for two main species of commercially available essential oils; namely chamomile and marjoram. Samples of the essential oil chamomile were taken from Rome, Germany and Morocco and the chemistry of each analysed by means of

GC-MS. The essential oil of chamomile originating from Rome was noted as being predominant in isobutyl angelate (34.6%) concentration; while the essential oils obtained from Germany and Morocco were determined as comprising predominantly in α-bisabolol oxide A (46.0%) and santolina alcohol (45.5%), respectively. For the essential oil of marjoram, samples were isolated from Spain and France with definitive differences noted in chemical makeup.

3.2.2. *L. angustifolia* essential oil chemistry

The full chemical analysis of the essential oil of *L. angustifolia* used in this study is given in Table 3.2.

Table 3.2 The chemical composition of the essential oil *L. angustifolia* under investigation.

RRI	Constituents	Percentage Abundance	RRI	Constituents	Percentage Abundance
1016	α-Pinene	0.1	1447	*cis*-Linalool oxide	0.2
1019	α-Thujene	t.a.*	1471	*cis*-Linalool oxide	0.1
1057	Camphene	0.1	1521	Camphor	0.3
1104	β -Pinene	t.a.	1541	Linalool	**31.4**
1159	Myrcene	0.2	1563	Linalyl acetate	**36.7**
1193	α-Terpinene	t.a.	1572	α-Cedrene	t.a.
1194	Limonene	0.1	1573	α-Santalene	0.4
1202	Eucalyptol	0.5	1584	α-Bergamotene	0.3
1232	β-*trans*-Ocimene	3.0	1602	Terpinen-4-ol	**14.9**
1242	γ-Terpinene	0.1	1665	β-Farnesene	1.4
1250	β-*cis*-Ocimene	2.1	1677	Lavandulol	1.2
1319	3-Octanone	0.4	1680	Cryptone	0.2
1270	*p*-Cymene	0.1	1701	α-Terpineol	0.3
1281	Terpinolene	t.a.	1702	Borneol	0.7
1331	Hexyl butyrate	0.1	1741	Carvone	t.a.
1372	Allo-ocimene	0.5	1763	γ-Cadinene	0.2
1376	1-Octenyl acetate	0.8	1797	Cuminaldehyde	0.1
1385	3-Octanol	0.1	1855	*p*-Cymen-8-ol	0.3
1411	*n*-Hexyl butyrate	0.3	2010	Caryophyllene epoxide	t.a.
1441	Hexyl-2-methylbutyrate	t.a.	2225	Thymol	0.1
				Total	**97.30%**

*t.a. = trace amounts, **bold** indicates major chemical constituents.

On analysis of the essential oil *L. angustifolia*, linalyl acetate (36.7%), linalool (31.4%) and terpinen-4-ol (14.9%) were identified as the major chemical constituents. The chemical composition of *L. angustifolia* has been studied extensively in literature, with congruency identified with regard to the major chemical constituents of *L. angustifolia* in the following studies; Daferera *et al.* (2000); Behnam *et al.* (2006); Roller *et al.* (2009) and Soković *et al.* (2010).

Although some level of similarity has been identified between data obtained in previously conducted research and the data of this study, slight variances in the chemical composition of *L. angustifolia* essential oil have also been noted in a number of other publications. One such study, conducted by Adam *et al.* (1998), determined the major constituents of this oil to be linalool (20.2%), linalyl acetate (18.6%) lavandulyl acetate (16.0%) and 1,8-cineole (13.1%), while a study performed by Hassiotis *et al.* (2010) reported linalool (20.1%), linalyl acetate (13.3%) and eucalyptol (12.4%) to be the major chemical constituents of *L. angustifolia.* Other studies show no congruency with major constituents varying completely, these studies include; Bayoub *et al.* (2010) and Alexopoulos *et al.* (2011) in which linalyl anthrilate (46.3%) and carvacrol (78.9%) are deemed the primary major chemical constituents of *L. angustifolia*, respectively.

This variation in essential oil chemistry may be due to the external, climatic factors previously outlined in Section 3.2.1. A study conducted by Lis-Balchin *et al*. (1998) further emphasises this point as two samples of *L. angustifolia* essential oil originating from varying regions of the world were analysed by GC-MS to determine possible correlations or deviances in essential oil chemistry dependant on the original location of plant harvest. The essential oil of *L. angustifolia* originating from France was identified as comprising of linalyl acetate (47.9%) and linalool (26.1%), while the essential oil obtained from Bulgaria was noted as being primarily comprised of linalyl acetate (79.8%). A study conducted by Hassiotis *et al*. (2010) aimed to identify possible differences in the chemical composition of *L. angustifolia* essential oil samples obtained from two different regions of Greece, as well as at four different intervals of the day. According to Hassiotis *et al*. (2010) the chemical composition of *L. angustifolia* harvested in Kato Sholari was comprised of linalyl acetate (30.6%) and linalool (29.6%); while the sample taken from Kilkis identified linalyl acetate (26.9%), linalool (16.8%) and 1, 8-cineole (15.6%) as the essential oils major chemical constituents. Further investigation determined that not only soil differences were responsible

for changes in chemical composition of the essential oils produced, but also the time at which harvest occurs. The sample of *L. angustifolia* taken from Kato Sholari was investigated further by determining the chemical composition of the essential oil after the plant had been harvested at 06h00, 10h00, 14h00 and 18h00. From the essential oil analysis it was determined that the concentration levels of linalyl acetate in the essential oil increases during the course of the day with 39.3% determined at the 06h00 sample and 47.7% determined for the 18h00 sample. The concentration of linalool however, decreased as the days harvesting progressed as sample obtained at 06h00 were determined as comprising of 34.4% linalool, while the 18h00 sample constituted 28.8% linalool.

The antimicrobial activity of essential oils has been attributed to the interactive effects of the chemical constituents (major and minor) in them and as such, slight variations in essential oil chemistry may impact on the antimicrobial effects. Therefore it is important to be cognisant of such factors when performing antimicrobial assays (Bassolé and Juliani, 2012).

3.3. Overview

The chemistry of the essential oils chosen for analysis in combination with *L. angustifolia* have been extensively studied and recorded previously. Of the 54 essential oil samples investigated, 50% have had the chemotype identified in this study previously investigated and reported on in two or more scientific studies, suggesting that these essential oils have been well characterised.

3.4. General conclusion

- Differences in essential oil chemotype were identified for the different parts of the plant (seed and root) for the essential oil *Angelica archangelica*.

- The essential oils of *Citrus medica limonum*, *Citrus sinensis* and *Mentha piperita* from varying origins demonstrated the same chemical constituents however, variances occurred in the percentage of the constituent in the essential oils.

- *Lavandula angustifolia* essential oil has been well characterised in previous literature.

CHAPTER 4
THE ANTIMICROBIAL PROPERTIES OF *L. ANGUSTIFOLIA* AND COMBINATIONS WITH SELECTED ESSENTIAL OILS

4.1. Introduction

Aromatherapy as defined by Buckle (2003) is the use of essential oils for therapeutic or medicinal purposes. In the practice of aromatherapy, essential oils are placed in combination in order to stimulate the mind, body and senses to bring about healing in a holistic manner (Shealy, 1998). The earliest use of essential oil blends can be traced back to the Egyptians where combinations of oils were used in the embalming process in order to protect the body against microbial decay (Hili, 2001).

Based on the aroma-therapeutic uses (antibiotic and antiseptic properties) of essential oils, *Lavandula angustifolia* has been combined with many other oils such as *Citrus aurantium*, *Anthemis nobilis*, *Salvia sclarea*, *Pelargonium odoratissimum*, *Citrus medica limonum*, *Citrus sinensis*, *Pogostemon patchouli*, *Rosmarinus officinalis*, *Cananga odorata*, *Citrus grandis*, *Origanum majorana*, *Juniperus virginiana* and *Eugenia caryophyllus* (Shealy, 1998). *L. angustifolia* has also been suggested in the use in combination with *Cympobogon martinii*, *J. virginiana*, *Salvia sclarea* and *Melaleuca alternifolia* (Hili, 2001) for the production of a favourably smelling essential oil blend. According to Curtis (1996), *L. angustifolia* can be used in combination with *C. medica limonum* as an antimicrobial agent against common urinary tract infections, however, no clinical study has been found to confirm this.

Only two scientific studies were identified to validate the use of *L. angustifolia* essential oil in combination with common aroma-therapeutic essential oils. One such study was conducted by Edwards-Jones *et al.* (2004) in which *L. officinalis* (now known as *L. angustifolia*) was placed in combination with *Melaleuca alternifolia*, *Pogostemon cablin*, *Pelargonium graveolens* and Citricidal™. Citricidal™ is a grapefruit seed extract, commercially available as an antibacterial agent. These products were placed in 1:1 combinations and tested against three strains of *S. aureus.* It was reported that *L. officinalis* in combination with

P. graveolens and *L. officinalis* in combination with *M. alternifolia* demonstrated increased inhibitory activity. The combination of *L. officinalis* and *M. alternifolia* essential oil however, was shown as having antagonistic antimicrobial effects when tested against the micro-organism methicillin-resistent *Staphylococcus aureus* (MRSA). Another study, conducted by Cassella (2002) aimed to identify the antimicrobial activity of *M. alternifolia* in combination with *L. officinalis* against the micro-organisms *Trichophyton rubrum* and *T. mentagrophytes* var. *interdigitale*. The data from this study confirmed that synergistic activity was evident when these oils were placed in combination. This chapter aims to investigate the antimicrobial properties of *L. angustifolia* independently and in combination with essential oils of antimicrobial importance.

4.2. Results and discussion

4.2.1. The antimicrobial activity of *L. angustifolia* essential oil

Initially, *L. angustifolia* essential oil was investigated for antimicrobial efficacy against 14 test micro-organisms (Table 4.1).

Table 4.1 The mean (n= 3) MIC values identified for *L. angustifolia* against 14 test micro-organisms.

Micro-organism	Type	MIC (mg/ml)
Methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 43300)	Gram-positive	2.00
Methicillin-resistant *Staphylococcus aureus* (Clinical strain 6437938)	Gram-positive	2.00
Methicillin-gentamicin resistant *Staphylococcus aureus* (MGRSA) (ATCC 33592)	Gram-positive	2.00
Staphylococcus aureus (ATCC 6538)	Gram-positive	2.00
Staphylococcus aureus (Clinical strain 6437938)	Gram-positive	2.00
Staphylococcus epidermidis (ATCC 2223)	Gram-positive	2.00
Staphylococcus epidermidis (Clinical strain BA 16)	Gram-positive	2.00
Vancomycin-resistant *Enterococcus faecalis* (ATCC 51299)	Gram-positive	2.00
Enterococcus faecalis (Clinical strain 6059015)	Gram-positive	2.00
Klebsiella pneumoniae (ATCC 13883)	Gram-negative	1.50
Pseudomonas aeruginosa (ATCC 27853)	Gram-negative	2.00
Cryptococcus neoformans (ATCC 11093)	Yeast	2.00
Candida tropicalis (ATCC 201380)	Yeast	0.75
Candida albicans (ATCC 10231)	Yeast	3.00
Ciprofloxacin (Positive control)	$0.20x10^{-3}$ to $0.60x10^{-3}$	
Amphotericin B (Positive control)	$0.60x10^{-3}$	

L. angustifolia essential oil obtained similar antimicrobial efficacies against most of the micro-organisms tested with an average MIC value of 2.00 mg/ml. As noteworthy activity for essential oils is accepted for MIC values less than or equivalent to 2.00 mg/ml (van Vuuren, 2008), it was identified that *L. angustifolia* essential oil obtained 90% noteworthy activity against all the micro-organisms tested. The micro-organism least susceptible to the effects of *L. angustifolia* essential oil was *C. albicans* with an MIC value of 3.00 mg/ml, while *C. tropicalis* was the micro-organism most susceptible with an MIC value of 0.75 mg/ml (Table 4.1).

In a study conducted by Hammer *et al.* (1999), *L. angustifolia* essential oil was tested against the yeast *C. albicans* and the MIC identified using the broth microdilution assay. In the study, it was identified that *L. angustifolia* essential oil obtained an average MIC value of 0.50% (v/v). According to Agarwal *et al*. (2010) antimicrobial activity is considered to be effective with MIC values ranging from 0.05% (v/v) to <0.15% (v/v). Moderate antimicrobial effects were determined for values ranging from >0.15% (v/v) to <1.00% (v/v), while poor antimicrobial effects are determined for MIC values of >1.00% (v/v). This study is therefore congruent with our research. A study conducted by Schwiertz *et al*. (2006) aimed to identify the antimicrobial effect of ten essential oils against a variety of bacterial and fungal vaginal strains, including *C. tropicalis.* Results showed that *L. angustifolia* demonstrated a MIC value of 1.0-2.5 µl/ml against this micro-organism, thus deeming it one of the more potent of the essential oils tested. This result is congruent with the findings generated in this study as it indicates the antimicrobial potential of this essential oil against *C. tropicalis.*

There have been a number of studies (Table 1.2) conducted on the antimicrobial activity of *L. angustifolia* essential oil against a wide variety of micro-organisms, however, many of them are no longer considered accurate as the method of disc diffusion was used to quantify antimicrobial activity (See comments made in Chapter 1, Section 1.7). Therefore, results from MIC methodology will only be considered when making comparative evaluations (Table 4.2). The results obtained from the varying MIC methodology of broth microdilution and agar dilution are considered comparable according to literature (Luber *et al*., 2003; Espinel-Ingroffa, 2006; Amsler *et al*., 2010). As such, literatures using these methods have been employed within this study for comparative purposes.

Table 4.2 Previous MIC studies conducted on the antimicrobial effects of *L. angustifolia* essential oil against the selected test pathogens of this study.

Micro-organism	Method	MIC	Reference
MRSA	Broth microdilution	0.50*	Nelson, 1997
MRSA - Clinical strain	No previous research identified		
MGRSA	No previous research identified		
Staphylococcus aureus	Broth microdilution	0.50*	Hammer *et al*., 1999
	Agar dilution	2.58‡	Mayaud *et al*., 2008
Staphylococcus aureus – Clinical strain	Broth microdilution	1.00*	Alexopoulos *et al*., 2011
Staphylococcus epidermidis	Broth microdilution	4.00†	Soković *et al*., 2010
Staphylococcus epidermidis – Clinical strain	No previous research identified		
Vancomycin-resistant *Enterococcus faecalis*	Broth microdilution	0.50‡	Nelson, 1997
Enterococcus faecalis – Clinical strain	No previous research identified		
Klebsiella pneumoniae	Agar dilution	2.00‡	Hammer *et al*., 1998
Pseudomonas aeruginosa	Agar dilution	No activity	Mayaud *et al*., 2008
	Broth microdilution	No activity	Hammer *et al*., 1999
Cryptococcus neoformans	No previous research identified		
Candida tropicalis		0.01-0.025*	Schwiertz *et al*., 2006
Candida albicans	Broth microdilution	0.50*	Hammer *et al*., 1999
	Broth microdilution	No activity	Agarwal *et al*., 2010

*MIC measured in mg/ml, †MIC measured in µg/ml, ‡MIC measured in % (v/v).

The bacteria identified as having been most affected by *L. angustifolia* essential oil, was *K. pneumoniae* as an average MIC value of 1.50 mg/ml was determined against this micro-organism. In a study conducted by Hammer *et al.* (1998), *L. angustifolia* essential oil was tested against the micro-organism *K. pneumoniae* using the agar dilution method to obtain an average MIC of 2.00% (v/v). *K. pneumoniae* was thus identified as the second most affected Gram-negative bacterium after *E. coli* against which an MIC value of 0.50 mg/ml was obtained. This study is congruent with that of Hammer *et al.* (1998).

For the class of Gram-positive bacteria, it was identified that *L. angustifolia* essential oil obtained an average MIC value of 2.00 mg/ml against all the micro-organisms investigated in this group. *L. angustifolia* obtained an average MIC value of 2.00 mg/ml against the Gram-positive bacterium methicillin-resistant *Staphylococcus aureus* (MRSA). Other previous studies have been conducted on the effects of *L. angustifolia* essential oil against this micro-organism (MIC of 0.50 mg/ml) (Nelson, 1997). Against the micro-organism *S. aureus*, *L. angustifolia* obtained an average MIC value of 2.00 mg/ml. Earlier scientific studies have also been conducted on the effects of *L. angustifolia* essential oil against the micro-organism

S. aureus, such as the study conducted by Hammer *et al.* (1999) in which an average MIC value of 0.50-1.00 mg/ml was observed.

A study conducted by Alexopoulos *et al.* (2011) was the only identifiable study in which *L. angustifolia* essential oil was tested against a clinical strain of *S. aureus*. This investigation is noteworthy as the micro-organism may have variances in its activity when compared to a laboratory strain due to the growing emergence of resistance. In the study conducted by Alexopoulos *et al.* (2011), *L. angustifolia* essential oil had a MIC value of 1.00 mg/ml.

For the micro-organism *S. epidermidis*, only one identifiable study has been conducted in which the effects of *L. angustifolia* essential oil could be documented against this micro-organism. A study conducted by Soković *et al.* (2010) identified *L. angustifolia* essential oil having an MIC value of 4.00 µg/ml against the micro-organism *S. epidermidis*. This value is significantly lower than that obtained in this study.

In this study *L. angustifolia* essential oil was identified as having an MIC of 2.00 mg/ml against the pathogen *P. aeruginosa.* Congruency was identified in the study conducted by Hammer *et al.* (1999) in which *L. angustifolia* essential oil obtained an MIC value of >2.00 mg/ml using the agar dilution method.

A study conducted by Nelson (1997) aimed to identify the antimicrobial activity of five essential oils against resistant bacterial strains such as MRSA and vancomycin resistant *Enterococcus faecalis* VREF. In the study, an average MIC value of 0.50% (v/v) was observed and found to be congruent with the results obtained in this study.

These studies further validate the antimicrobial potential of *L. angustifolia* essential oil as well as augment its popularity in aromatherapy.

4.2.2. The antimicrobial activity of other common aroma-therapeutic essential oils

The 54 essential oils selected for combination analysis with *L. angustifolia* were tested against three test micro-organisms, *C. albicans*, *S. aureus* and *P. aeruginosa.* Due to the large number of samples investigated, one micro-organism per class was chosen for further

investigation. *S. aureus* was selected as a representative of the Gram-positive bacteria, whereas *P. aeruginosa* was chosen to represent the Gram-negative bacteria and *C. albicans* the yeasts (Table 4.3).

Table 4.3 The mean (n= 3) MIC values identified for the essential oils investigated.

Essential oil	MIC (mg/ml)			Previous research conducted
	C. albicans **(ATCC 10231)**	***S. aureus*** **(ATCC 6538)**	***P. aeruginosa*** **(ATCC 27858)**	
Abies balsamea	**2.00**	3.00	**2.00**	Pichette *et al*., 2006
Andropogon muricatus	**1.75**	**0.75**	**1.50**	Prashanth *et al*., 2011
Angelica archangelica (seed)	**2.00**	**1.75**	**2.00**	Janssen *et al*., 1986
Angelica archangelica (root)	**2.00**	**2.00**	**2.00**	
Anthemis nobilis	3.00	16.00	**2.00**	Duarte *et al*., 2005
Artemisia dracunculus	**2.00**	3.00	**2.00**	Benli *et al*., 2007; Mohsenzadeh, 2007;
Canarium luzonicum	3.00	3.00	**2.00**	No previous research identified
Cananga odorata (heads)	**2.00**	4.00	**1.50**	Janssen *et al*., 1986; Hammer *et al*., 1999
Cananga odorata	**2.00**	**2.00**	3.00	
Carum carvi	**2.00**	**2.00**	**2.00**	Janssen *et al*., 1986; de Martino *et al*., 2009
Cedrus atlantica	4.00	4.00	3.00	Hammer *et al*., 1999
Cinnamomum zeylanicum	**2.00**	**2.00**	**1.50**	Pozzatti *et al*., 2010
Citrus aurantium	**2.00**	4.00	**2.00**	Fabio *et al*., 2007; Hammer *et al*., 1999
Citrus grandis	4.00	4.00	**2.00**	No previous research identified
Citrus medica limonum	**2.00**	3.00	**2.00**	Fabio *et al*., 2007; Gupta *et al*., 2008
Citrus medica limonum (Argentina)	**2.00**	6.00	**2.00**	
Citrus paradisi	**2.00**	3.00	**1.50**	No previous research identified
Citrus sinensis	**2.00**	**2.00**	**2.00**	Prabuseenivasan *et al*., 2006; Fabio *et al*., 2007; Espina *et al*., 2011
Citrus sinensis (Brazil)	**2.00**	6.00	**2.00**	Prabuseenivasan *et al*., 2006; Fabio *et al*., 2007; Espina *et al*., 2011
Citrus sinensis (Florida)	**2.00**	4.00	**2.00**	
Commiphora myrrha	4.00	**2.00**	4.00	Hanùs *et al*., 2005; de Rapper *et al*., 2012
Cupressus sempervirens	4.00	12.00	**2.00**	Fabio *et al*., 2007; Hammer *et al*., 1999; Hassanzadeh *et al*., 2010

Table 4.3 continued The mean (n= 3) MIC values identified for the essential oils investigated.

Essential oil	MIC (mg/ml)			Previous research conducted
	C. albicans **(ATCC 10231)**	***S. aureus*** **(ATCC 6538)**	***P. aeruginosa*** **(ATCC 27858)**	
Cymbopogon citratus	**2.00**	**1.67**	**1.50**	Irkin and Korukluoglu, 2009; Bassolé *et al*., 2011
Cympobogon martinii	**0.75**	**1.50**	**1.50**	Hammer *et al*., 1999; Delespaul *et al*., 2000; Duarte *et al*., 2005
Cymbopogon nardus	**0.75**	4.00	**1.50**	Hammer *et al*., 1999
Daucus carota	3.00	**2.00**	3.00	Hammer *et al*., 1999
Eucalyptus globulus	**1.50**	4.00	3.00	Gupta *et al*., 2008; Mayaud *et al*., 2008
Eugenia caryophyllus	**0.50**	**1.50**	**1.50**	Agarwal *et al*., 2008; Agarwal *et al*., 2010
Foeniculum dulce	**2.00**	**2.00**	3.00	Shahat *et al*., 2011
Hyssopus officinalis	**1.00**	3.00	**2.00**	Mazzanti *et al*., 1998
Juniperus communis (berries)	**2.00**	3.00	**2.00**	Adams, 1987; Eller *et al*., 2010
Juniperus virginiana (China)	**1.50**	**2.00**	**2.00**	
Juniperus virginiana (Virginia)	**0.75**	**1.50**	**2.00**	
Laurus nobilis	**0.75**	**0.83**	2.67	Dadalioğlu and Evrendilek, 2004; Al-Hussaini and Mahasneh, 2011
Litsea cubeba	6.00	**1.50**	**1.50**	Wang and Liu, 2010
Matricaria chamomilla	**0.50**	**1.50**	4.00	Agarwal *et al*., 2008; Agarwal *et al*., 2010
Melaleuca alternifolia	**1.50**	8.00	**2.00**	Cox *et al*., 2001
Melaleuca viridiflora	**1.75**	**2.00**	**2.00**	Ramanoelina *et al*., 1987
Mentha piperita	**1.50**	3.00	**2.00**	Hammer *et al*., 1999; Duarte *et al*., 2005; Fabio *et al*., 2007
Mentha piperita (America)	**2.00**	4.00	**2.00**	
Mentha piperita (China)	**2.00**	4.00	**2.00**	
Myrtus communis	**1.50**	**2.00**	**2.00**	Mansouri *et al*., 2001; Özcan and Erkmen, 2001
Ocimum basilicum	**1.00**	**1.50**	2.67	Hammer *et al*., 1999; Hussain *et al*., 2007
Origanum majorana	**2.00**	**2.00**	**2.00**	Hammer *et al*., 1999; Lixandru *et al*., 2010
Pelargonium odoratissimum	**0.75**	**1.50**	**2.00**	Andrade *et al*., 2011
Piper nigrum	**2.00**	**2.00**	**2.00**	Hammer *et al*., 1999
Pogostemon patchouli	**1.50**	**1.50**	**2.00**	Hammer *et al*., 1999

Table 4.3 continued The mean (n= 3) MIC values identified for the essential oils investigated.

Essential oil	MIC (mg/ml)			Previous research conducted
	***C. albicans* (ATCC 10231)**	***S. aureus* (ATCC 6538)**	***P. aeruginosa* (ATCC 27858)**	
Rosmarinus angustifolia	**2.00**	4.00	**2.00**	Hammer *et al*., 1999; Fabio *et al*., 2007; Lixandru *et al*., 2010; Al-Hussaini and Mahasneh, 2011
Rosmarinus officinalis	**2.00**	4.00	**2.00**	
Salvia sclarea	**0.88**	**2.00**	3.50	Hammer *et al*., 1999; Peana *et al*., 1999
Santalum album	**2.00**	**0.25**	**0.50**	Hammer *et al*., 1998; Hammer *et al*., 1999
Styrax benzoin	**2.00**	**2.00**	3.00	No previous research identified
Tagetes minuta	**2.00**	4.00	**1.50**	Héthélyi *et al*., 1986
Thymus vulgaris	**1.00**	3.33	**2.00**	Hammer *et al*., 1999; Fabio *et al*., 2007; Lixandru *et al*., 2010
Ciprofloxacin Positive control	NR*	$0.30x10^{-3}$	$0.60x10^{-3}$	
Amphotericin B Positive control	$0.60x10^{-3}$	NR*	NR*	

Bold= noteworthy antimicrobial activity (≤2.00 mg/ml), *NR= Not relevant control for pathogen.

Of the 54 essential oil samples tested, 72.2% demonstrated noteworthy antimicrobial activity. These essential oils presented the greatest antimicrobial effect against the micro-organism *P. aeruginosa* with 100.0% of the samples exhibiting moderate to noteworthy effects (Figure 4.1).

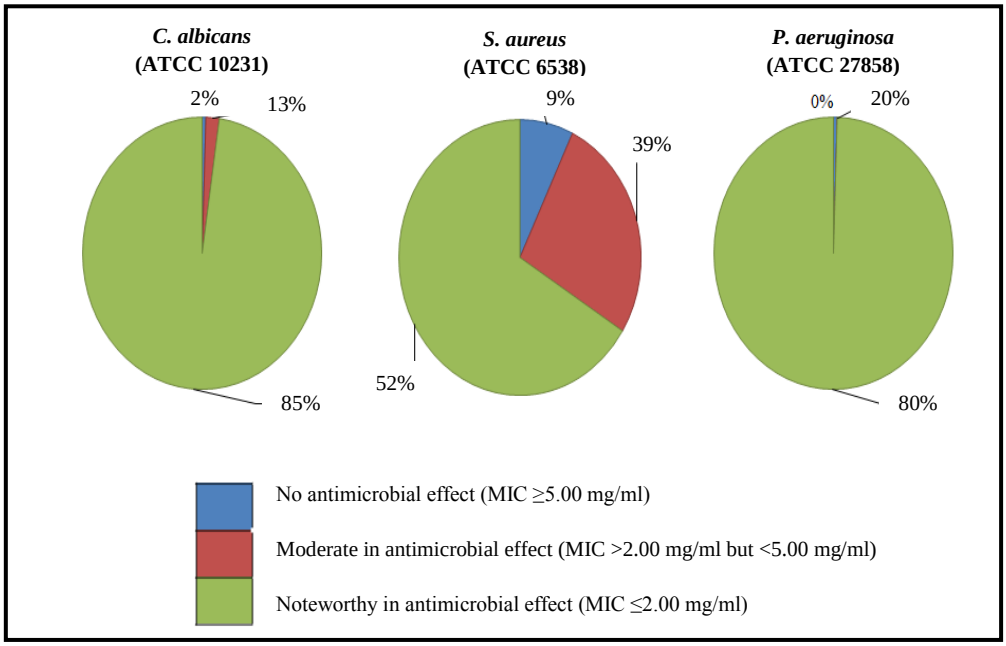


Figure 4.1 The percentage noteworthy antimicrobial activity of the 54 essential oil samples investigated against *C. albicans*, *S. aureus* and *P. aeruginosa*.

Moderate antimicrobial activity was classified for essential oils that exhibit MIC values of greater than 2.00 mg/ml and less than 5.00 mg/ml. Essential oils with MIC values of more than or equivalent to 5.00 mg/ml are considered as having no antimicrobial effect. With this in mind, only four essential oils (*Anthemis nobilis*, *Citrus medica limonum* (Argentina), *Citrus sinensis* (Brazil) and *Cupressus sempervirens*) demonstrated a lack of antimicrobial effect against the micro-organism *S. aureus*.

The MIC results determined for the essential oils against *C. albicans* varied between 0.50 mg/ml to 6.00 mg/ml (Figure 4.2). The essential oils *Matricaria chamomilla* and *Eugenia caryophyllus* obtained the best overall antimicrobial activity against *C. albicans* with an average MIC of 0.50 mg/ml. The essential oil of *M. chamomilla* has been indicated in the treatment of acne, cold sores, gum infections, boils and yeast infections due to its assumed general anti-infective and bactericidal activity (Sellar, 1992; Lawless, 1995; Curtis, 1996 and Shealy, 1998). *E. caryophyllus* essential oil has also been predominantly indicated in the treatment of yeast based infection such as thrush, while also indicted for use against acne and topical skin infections (Lawless, 1995; Curtis, 1996 and Hili, 2001).

The antimicrobial activity of *M. chamomilla* has been previously reported with strong antimicrobial effects determined. In a study conducted by Abdoul-Latif *et al*. (2011), *M. chamomilla* was investigated for antimicrobial effects against 15 micro-organisms, including *C. albicans* (ATCC 10231). From the study it was determined that *M. chamomilla* demonstrated an antimicrobial effect of 1.00 mg/ml against this pathogen, which is congruent with the findings of this research. *M. chamomilla* further demonstrated noteworthy antimicrobial activity (MIC values ranging from 1.00 mg/ml to 2.00 mg/ml) against the micro-organisms *Listeria innocua* (LMG 1135668), *Salmonella enterica* (CIP 105150), *Shigella dysenteria* (CIP 5451), *S. aureus* (ATCC 9244), *Staphylococcus camorum* (LMG 13567) and clinical strains of *C. albicans*, *Aspergillus niger*, *P. aeruginosa* and *Streptococcus pyogenes*. A study performed by Agarwal *et al*. (2010) determined that *E. caryophyllus* presented moderately effective antimicrobial activity against *C. albicans* with an MIC value of 0.30% (v/v). This result is congruent with the findings generated in this study (Table 4.3).

E. caryophyllus has also demonstrated significant antibacterial activity with MIC values ranging from 1.60 mg/ml to 6.40 mg/ml against the micro-organisms *E. coli* (ATCC 25922), *K. pneumoniae* (ATCC 15380), *P. aeruginosa* (ATCC 27853), *P. vulgaris* (MTCC 1771),

B. cereus (MTCC 441) and *S. aureus* (ATCC 25923) (Prabuseenivasan *et al*., 2006).

Santalum album was identified as the essential oil with the greatest antimicrobial effect against the micro-organism *S. aureus* with an MIC of 0.25 mg/ml, and *P. aeruginosa* with an MIC value of 0.50 mg/ml (Figure 4.2). In aromatherapy literature *S. album* essential oil is considered to have potent antimicrobial effects against a plethora of conditions ranging from a general sore throat and superficial skin infection, to sexually transmitted diseases such as gonorrhoea (Sellar, 1992 and Lawless, 1995). A study conducted by Hammer *et al.* (1998) determined the MIC (0.06 mg/ml) of *S. album* against *C. albicans* using the agar dilution method. In a later study (Hammer *et al.*, 1999) in which *S. album*, originating from Australia also demonstrated the best antimicrobial activity for *C. albicans* when compared with 52 other essential oils. No antimicrobial efficacy was noted against *P. aeruginosa* (MIC value of >2.00 mg/ml), while against *S. aureus*, very good antimicrobial activity was observed (MIC value of 0.12 mg/ml). These studies are congruent with the findings reported here; however greater sensitivity was noted against the micro-organism *P. aeruginosa* when evaluated in this study.

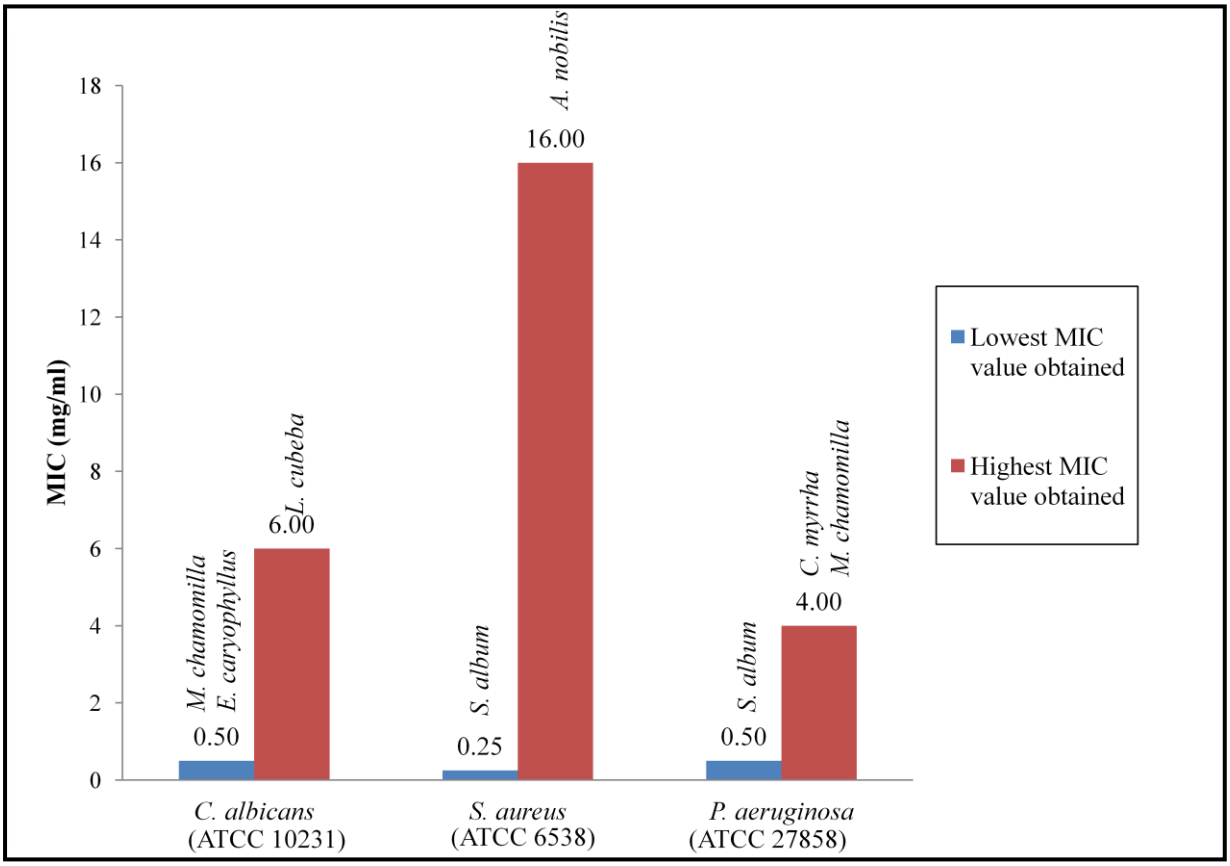


Figure 4.2 The highest and lowest MIC values obtained of the 54 essential oil samples investigated against *C. albicans*, *S. aureus* and *P. aeruginosa*.

The antimicrobial effect of an essential oil is influenced by a number of factors. One such factor is the chemical composition of an essential oil whereby interactions between the comprised major and minor chemical constituents may play a role (Bassolé and Juliani, 2012). Essential oil composition in turn is affected by a number of other factors, which were previously discussed in detail in Chapter 3. One factor that needs to be taken into consideration is the origin of the essential oil as this affects the chemical composition and in turn the possibility of MIC variations. A selection of essential oils in this study, *Mentha piperita*, *Juniperus virginiana*, *Citrus medica limonum* and *Citrus sinensis*, were obtained from varying regions of the world and their antimicrobial effects determined. It was noted that similarities in antimicrobial effect were demonstrated. This may be due to a greater similarity in essential oil composition. This phenomenon of inconsistencies in antimicrobial effects of essential oils of the same species has been extensively studied with numerous researchers attributing antimicrobial variability to origin and chemistry (Lis-Balchin *et al*., 1998; Milos *et al*., 2001; van Vuuren, 2008; Hassiotis *et al*., 2010). A study conducted by van Vuuren (2007) sought to identify possible differences in essential oil antimicrobial effects of samples collected in various regions of South Africa. One such essential oil was *Heteropyxis natalensis* collected from seven different locations including Johannesburg, Nelspruit and Lagalametse. These varying samples were investigated against five micro-organisms and the antimicrobial effects determined by means of MIC analysis. A large variation in antimicrobial effect was determined against the micro-organism *Enterococcus faecalis* with MIC values ranging from 16.00 mg/ml for the sample collected in Cullinan to 3.00 mg/ml for the sample originating from Lagalametse.

4.2.3. The antimicrobial activity of *L. angustifolia* essential oil in combination with common aroma-therapeutic essential oils at a 1:1 ratio

When the 54 essential oil samples were placed in combination with *L. angustifolia* at a 1:1 ratio, ΣFIC values where calculated for each combination (Table 4.4).

Table 4.4 The mean MIC (n=3) and ΣFIC values of the essential oil combinations investigated.

Essential oil combinations	Mean MIC (mg/ml) and ΣFIC					
	***C. albicans* (ATCC 10231)**		***S. aureus* (ATCC 6538)**		***P. aeruginosa* (ATCC 27858)**	
	MIC	**ΣFIC**	**MIC**	**ΣFIC**	**MIC**	**ΣFIC**
Lavandula angustifolia + Abies balsamea	**1.50**	0.63	6.00	2.50	**1.00**	0.52
Lavandula angustifolia + Andropogon muricatus	**1.00**	**0.45**	**1.00**	0.92	**2.00**	1.02
Lavandula angustifolia + Angelica archangelica (seed)	**1.00**	**0.42**	**2.00**	1.07	**1.00**	0.67
Lavandula angustifolia + Angelica archangelica (root)	**2.00**	0.83	4.00	2.00	**1.00**	0.75
Lavandula angustifolia + Anthemis nobilis	**1.00**	**0.33**	3.00	0.84	**1.00**	0.54
Lavandula angustifolia + Artemisia dracunculus	**1.00**	**0.42**	4.00	1.67	**1.00**	0.51
Lavandula angustifolia + Canarium luzonicum	**0.75**	**0.25**	8.00	3.33	**1.00**	0.53
Lavandula angustifolia + Cananga odorata (heads)	**2.00**	0.83	3.00	1.13	**2.00**	1.02
Lavandula angustifolia + Cananga odorata	3.00	1.25	3.00	1.50	**2.00**	1.02
Lavandula angustifolia + Carum carvi	**1.00**	**0.42**	**2.00**	1.00	**1.00**	0.56
Lavandula angustifolia + Cedrus atlantica	**1.00**	**0.29**	**2.00**	0.75	**1.00**	0.55
Lavandula angustifolia + Cinnamomum zeylanicum	**1.00**	**0.40**	**1.00**	**0.50**	**1.00**	0.53
Lavandula angustifolia + Citrus aurantium	**1.00**	**0.42**	3.00	1.13	**1.00**	0.51
Lavandula angustifolia + Citrus grandis	**2.00**	0.58	3.00	1.13	**1.00**	0.52
Lavandula angustifolia + Citrus medica limonum	**1.00**	**0.42**	6.00	2.50	**1.00**	0.52
Lavandula angustifolia + Citrus medica limonum (Argentina)	**1.50**	0.63	5.00	1.67	**1.00**	0.52
Lavandula angustifolia + Citrus paradisi	**1.00**	**0.42**	4.00	1.67	**1.00**	0.52
Lavandula angustifolia + Citrus sinensis	**2.00**	0.83	4.00	2.00	**1.00**	0.51
Lavandula angustifolia + Citrus sinensis (Brazil)	**2.00**	0.83	**2.00**	0.67	**1.00**	0.51
Lavandula angustifolia + Citrus sinensis (Florida)	**1.00**	**0.42**	**1.00**	**0.38**	**1.00**	0.51
Lavandula angustifolia + Commiphora myrrha	**1.00**	**0.29**	**2.00**	1.00	**2.00**	1.03
Lavandula angustifolia + Cupressus sempervirens	**0.50**	**0.15**	**2.00**	0.58	**1.00**	0.53
Lavandula angustifolia + Cymbopogon citrates	**0.50**	6.67	**1.00**	0.55	**1.00**	0.52
Lavandula angustifolia + Cympobogon martini	**1.00**	0.83	**1.00**	0.58	**1.00**	0.51
Lavandula angustifolia + Cymbopogon nardus	**0.75**	**0.42**	**2.00**	0.75	**1.00**	0.53
Lavandula angustifolia + Daucus carota	**1.50**	**0.50**	**1.00**	**0.50**	**1.00**	0.56
Lavandula angustifolia + Eucalyptus globulus	**0.75**	**0.38**	4.00	1.50	**1.00**	0.53
Lavandula angustifolia + Eugenia caryophyllus	**0.50**	0.58	**2.00**	1.17	**1.00**	0.53
Lavandula angustifolia + Foeniculum dulce	**1.00**	**0.45**	4.00	2.00	**1.00**	0.52
Lavandula angustifolia + Hyssopus officinalis	**0.50**	**0.33**	4.00	1.67	**1.00**	0.52
Lavandula angustifolia + Juniperus communis (berries)	**0.50**	**0.21**	3.00	1.25	**1.00**	0.52
Lavandula angustifolia + Juniperus virginiana (China)	**1.00**	**0.50**	**1.00**	**0.50**	**1.00**	0.55
Lavandula angustifolia + Juniperus virginiana (Virginia)	**1.00**	0.83	**1.00**	0.58	**1.00**	0.54
Lavandula angustifolia + Laurus nobilis	**1.00**	0.83	**2.00**	1.70	**1.00**	0.60
Lavandula angustifolia + Litsea cubeba	**0.75**	**0.19**	**2.00**	1.17	**1.00**	0.52

Table 4.4 The mean MIC (n=3) and ΣFIC values of the essential oil combinations investigated.

Essential oil combinations	Mean MIC (mg/ml) and ΣFIC					
	***C. albicans* (ATCC 10231)**		***S. aureus* (ATCC 6538)**		***P. aeruginosa* (ATCC 27858)**	
	MIC	**ΣFIC**	**MIC**	**ΣFIC**	**MIC**	**ΣFIC**
Lavandula angustifolia + Matricaria chamomiua	**1.00**	1.17	**2.00**	1.17	**1.00**	0.54
Lavandula angustifolia + Melaleuca alternifolia	**1.00**	**0.50**	**2.00**	0.63	**1.00**	0.51
Lavandula angustifolia + Melaleuca viridiflora	**2.00**	0.90	4.00	2.00	**1.00**	0.51
Lavandula angustifolia + Mentha piperita	**1.00**	**0.50**	3.00	1.25	**2.00**	1.02
Lavandula angustifolia + Mentha piperita (America)	**1.50**	0.63	**2.00**	0.75	**2.00**	1.02
Lavandula angustifolia + Mentha piperita (China)	**1.50**	0.63	**2.00**	0.75	**1.00**	0.51
Lavandula angustifolia + Myrtus communis	**1.00**	**0.50**	**2.00**	4.00	**1.00**	0.51
Lavandula angustifolia + Ocimum basilicum	**1.00**	0.67	**1.00**	0.58	**1.00**	0.63
Lavandula angustifolia + Origanum marjorana	**1.00**	**0.42**	**2.00**	4.00	**1.00**	0.52
Lavandula angustifolia + Pelargonium odoratissimum	**1.25**	1.04	**2.00**	1.17	**1.00**	0.52
Lavandula angustifolia + Piper nigrum	**1.00**	**0.42**	**2.00**	1.00	**1.00**	0.57
Lavandula angustifolia + Pogostemon patchouli	**1.00**	**0.50**	**2.00**	1.17	**1.00**	0.51
Lavandula angustifolia + Rosmarinus angustifolia	**1.00**	**0.42**	**2.00**	0.75	**2.00**	1.02
Lavandula angustifolia + Rosmarinus officinalis	**1.00**	**0.42**	**2.00**	0.75	**1.00**	0.51
Lavandula angustifolia + Salvia sclarea	**1.00**	0.73	**2.00**	1.00	**1.00**	0.51
Lavandula angustifolia + Santalum album	**1.00**	**0.42**	**1.00**	2.25	**1.00**	0.51
Lavandula angustifolia + Styrax benzoin	**1.00**	**0.42**	**2.00**	1.00	**1.00**	0.58
Lavandula angustifolia + Tagetes minuta	**1.00**	**0.42**	**2.00**	0.75	**1.00**	0.51
Lavandula angustifolia + Thymus vulgaris	**1.00**	0.67	**1.00**	**0.40**	**1.00**	0.51
Ciprofloxacin control	NR*	NR*	0.30×10^{-3}	NR*	0.60×10^{-3}	NR*
Amphotericin B control	0.60×10^{-3}	NR*	NR*	NR*	NR*	NR*

Bold= noteworthy antimicrobial activity/synergy, highlighted= most promising combinations, NR*= Not relevant control for pathogen.

When the 54 essential oil samples were placed in combination with *L. angustifolia*, it was noted that 23.5% of the combinations were synergistic, 52.5% additive, 23.5% non-interactive and 0.5% antagonistic (Figure 4.3)

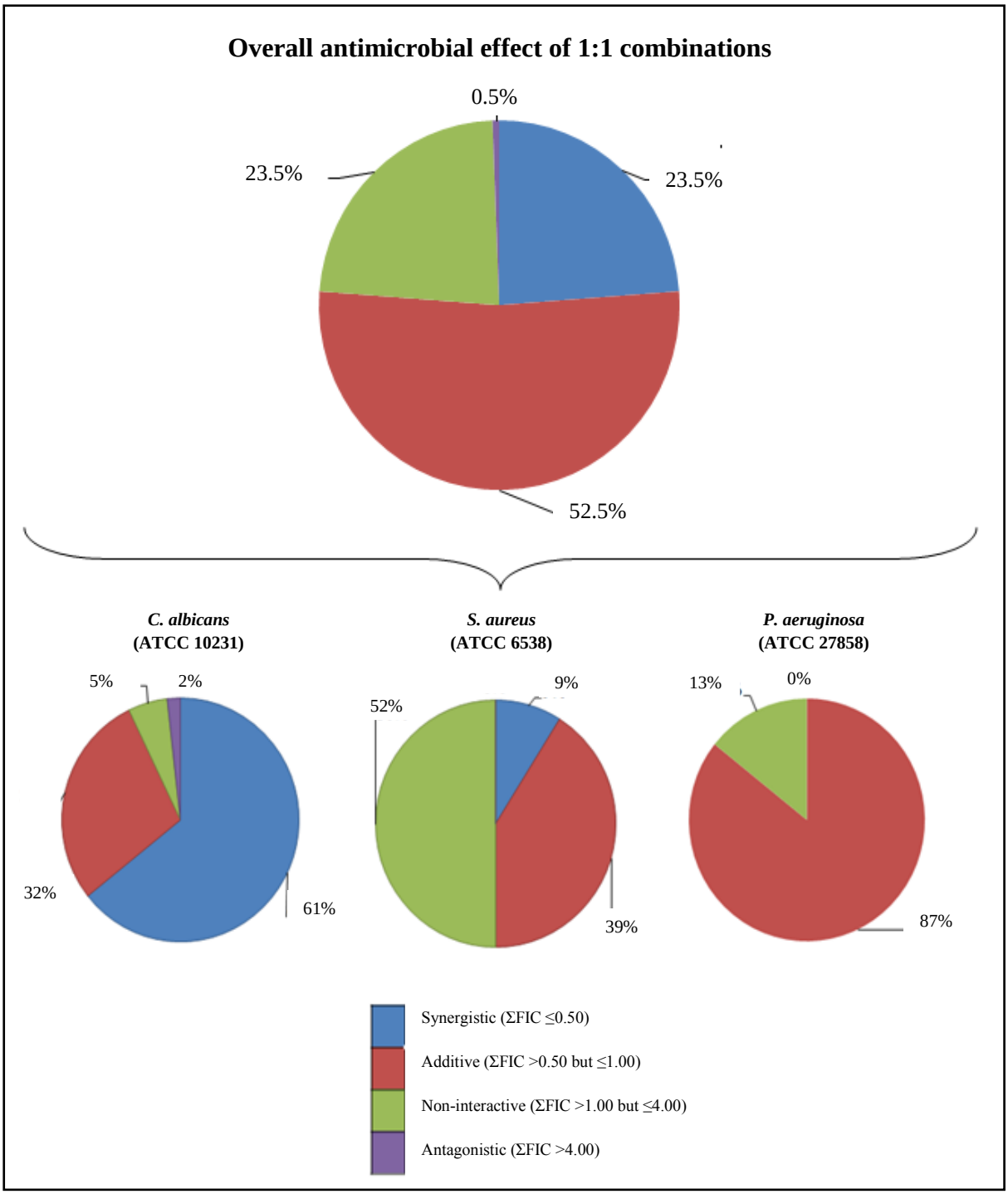


Figure 4.3 The antimicrobial interactions determined for the essential oil combinations against *C. albicans*, *S. aureus* and *P. aeruginosa*.

Against the micro-organism *C. albicans*, it was identified that the predominant effect of the essential oils when placed in combination, was synergy (61%). Against the micro-organism *S. aureus* a mainly non-interactive effect (52%) was observed for the combinations evaluated,

while against *P. aeruginosa* it was noted that the majority of essential oils in combination showed an additive effect (87%).

Of all the combinations evaluated, the most promising synergistic interaction was identified for the combination of *L. angustifolia* and *C. sempervirens* against the micro-organism *C. albicans* in which a ΣFIC value of 0.15 was obtained. Previous to this study, no research has been conducted on *L. angustifolia* in combination with *C. sempervirens* essential oil against the micro-organism *C. albicans*, however, *C. sempervirens* has been extensively studied individually. A study conducted by Hassanzadeh *et al.* (2010) aimed to identify the antimicrobial activity of Spanish *C. sempervirens* leaf essential oil against a variety of micro-organisms, including *C. albicans*. The study reported that *C. sempervirens* essential oil demonstrated noteworthy antimicrobial activity against *C. albicans* with an average MIC value of 0.625 mg/ml. This value is far lower than that obtained in the present study (MIC value of 4.00 mg/ml); however it does confirm the antimicrobial potential of this essential oil.

From the 54 essential oils combinations analysed, four essential oils in combination with *L. angustifolia* have been identified as having noteworthy and synergistic antimicrobial activity against more than one of the micro-organisms tested. These combinations include *L. angustifolia* in combination with *Cinnamomum zeylanicum, Citrus sinensis, Daucus carota* or *Juniperus virginiana.* The full chemical profiles of these essential oils were determined (Appendix D) in order to confirm the purity and specific chemotype of essential oil used in the present study.

In a study conducted by Pozzatti *et al.* (2010), *C. zeylanicum* essential oil was tested against *C. albicans* to identify its antimicrobial effect using the minimum inhibitory concentration assay. The MIC obtained was 0.40 mg/ml, which is far more effective than our study, which obtained an MIC value of 2.00 mg/ml. This inconsistency may be due to the difference in strains of *C. albicans* used in each study, as well as the part of the plant used for essential oil production which is not specified in the Pozzatti *et al.* (2010) study. It has been identified that *C. zeylanicum* bark essential oil appears to be more effective than the *C. zeylanicum* leaf essential oil, as is used in this study. Many other noteworthy activities have been found for *C. zeylanicum* essential oil against other micro-organisms (Deans and Ritchie, 1987; Quale *et al.*, 1996; Shahverdi *et al.*, 2007; Mayaud *et al.*, 2008; Reichling *et al.*, 2009; Jayaprakasha and Rao, 2011; Nanasombat and Wimuttigosol, 2011). The antimicrobial effects of

C. zeylanicum essential oil were shown to increase slightly in the presence of *L. angustifolia* (mean MIC value of 1.83 mg/ml to 1.00 mg/ml in combination). Previous to this study, no study has been conducted on *L. angustifolia* in combination with *C. zeylanicum* essential oil.

In a study conducted by Hammer *et al*. (1999) *D. carota* essential oil was investigated for its antimicrobial effects against a number of micro-organisms. According to Hammer *et al*. (1999), it was identified that *D. carota* demonstrated no antimicrobial effect against the micro-organisms investigated with exception to *C. albicans* and *E. faecalis* against which an MIC value of 2.00 % (v/v) . This is congruent with our findings (Table 4.4). When placed in combination with *L. angustifolia* essential oil it is noted that the antimicrobial effects of *D. carota* oil increase two fold (mean MIC value of 2.67 mg/ml to 1.16 mg/ml in combination). A study conducted by Prabuseenivasan *et al.* (2006) tested *C. sinensis* essential oil against the micro-organism *S. aureus* and the antimicrobial effect identified using a MIC assay. *C. sinensis* essential oil demonstrated a poor effect with an MIC of >12.80 mg/ml. A study conducted by Espina *et al.* (2011) aimed to test three essential oil samples against a variety of test micro-organisms, including *S. aureus. C. sinensis* essential oil was shown to obtain an MIC of 5.00 mg/ml which is congruent with that found in this study. Other studies have been conducted on the use of *C. sinensis* essential oil by means of other antimicrobial assays (Fabio *et al.*, 2007), as well as against a variety of other micro-organisms (O'Bryan *et al.*, 2008; Chao *et al.*, 2008). When placed in combination with *L. angustifolia* essential oil, the antimicrobial effects of *C. sinensis* oil improved almost three fold (mean MIC value of 2.67 mg/ml to 1.00 mg/ml in combination).

Very little is known of the antimicrobial properties of *J. virginiana* oil as it is often disfavoured over *Cedrus atlantica* oil due to its believed inferior safety and efficacy profile (Clarke, 2008). When investigated individually, *J. virginiana* essential oil demonstrated noteworthy antimicrobial effects against the micro-organisms tested with a mean MIC value of 1.83 mg/ml. Previous to this study, no study has been conducted on *L. angustifolia* in combination with *J. virginiana* essential oil.

From the data obtained on the antimicrobial efficacy of these essential oils in combination with *L. angustifolia*, it can be determined that 76% of the combinations have demonstrated positive interactive effects. This outcome lends some credibility to combining these essential oils in the field of aromatherapy.

4.2.4. The antimicrobial effect of *L. angustifolia* essential oil in combination with common aroma-therapeutic essential oils at variable ratios

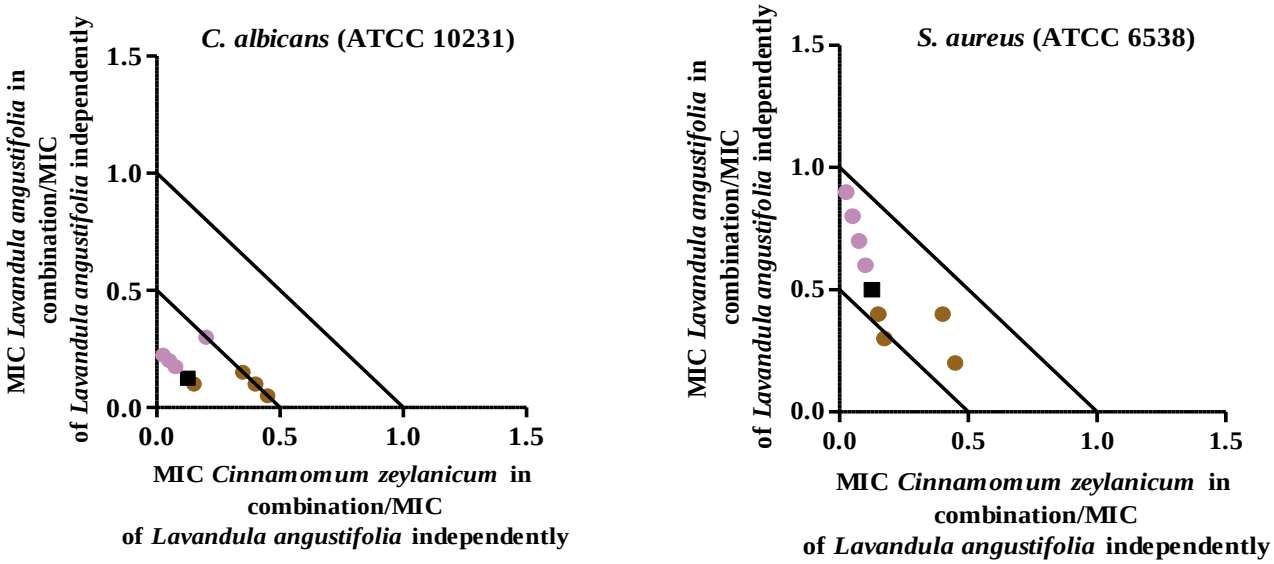
The art of using essential oils in combination, or blending, has been incorporated in aromatherapeutic practice for many years with no scientific support. The practice of blending essential oils is based primarily on the presumed synergistic therapeutic effect as well as the element of combined smell, with individual aromatherapists encouraged to develop their own combinations through practise (McGilvery and Reed, 1995). Due to this inconsistent means of mixing essential oils in aromatherapy for antimicrobial purposes, it is important to determine if a possible variation in antimicrobial activity exists when the essential oils are placed in varying ratios of concentration. The four essential oil combinations (*L. angustifolia* with either *D. carota, J. virginiana, C. zeylanicum* or *C. sinensis*) that demonstrated synergistic antimicrobial activity against both *C. albicans* and *S. aureus* were investigated further to determine if varying ratio concentrations of the essential oils in the combinations would demonstrate alternate interactive properties.

From the aromatherapy literature used to identify these combinations (Sellar, 1992; Lawless, 1995; Curtis, 1996; Shealy, 1998; Hili, 2001; Buckle, 2003; Lawrence, 2005), no specific formula could be determined for the blending of these essential oils for the desired therapeutic purpose.

***C. zeylanicum* and *L. angustifolia* in combination:** has been indicated as a general antimicrobial combination for the treatment of topical infections (Shealy, 1998).
L. angustifolia when combined with *C. zeylanicum* displayed the greatest synergistic effect of all oils studied when varied ratios of these two oils were investigated against the micro-organism *C. albicans* (Figure 4.4). For this combination it was determined that regardless of the ratios of the essential oils used in the combination, synergy would be achieved against *C. albicans*, however, at certain ratios a more potent effect is achieved. This combination should ideally be blended in equal ratios or in ratios higher in *L. angustifolia* concentration.

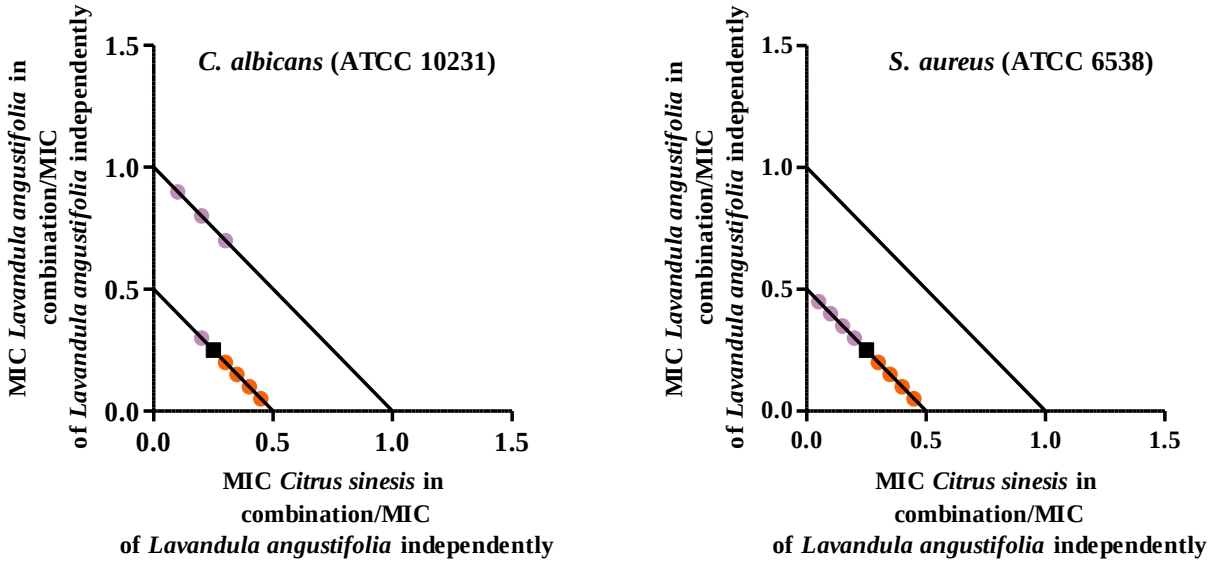
Against the micro-organism *S. aureus*, a predominant additive antimicrobial effect was noted, with one ratio (*L. angustifolia*: *C. zeylanicum*, 3:7) indicating synergy. A greater antibacterial effect was noted where the concentration of *C. zeylanicum* in the combination was dominant. It should however be stated that when the concentration of *C. zeylanicum* to *L. angustifolia*

essential oil reaches 8:2 the antimicrobial effect decreased in potency. For anti-staphylococcal purposes this essential oil combination should be ideally mixed in a ratio of *L. angustifolia*: *C. zeylanicum*, 3:7.



Figures 4.4 Isobologram representation of *Cinnamomum zeylanicum* essential oil in combination with *L. angustifolia* essential oil at various combination ratios. ⬭ Indicates *L. angustifolia* in majority; ⬭ indicates *C. zeylanicum* in majority and ■ indicates 1:1 ratio.

***C. sinensis* and *L. angustifolia* in combination:** has been indicated in the treatment of respiratory related infections. The antimicrobial efficacy of *C. sinensis* has been indicated for *C. albicans* related infections (Lawless, 1995; Shealy, 1998; Hili, 2001). According to previous literature (Prabuseenivasan *et al.*, 2006; O'Bryan *et al.*, 2008; Chao *et al.*, 2008), *C. sinensis* has proven to have a poor antimicrobial effect when applied independently. When *L. angustifolia* was combined with *C. sinensis* in various ratios a predominantly synergistic antimicrobial effect was identified, especially in concentrations where *C. sinensis* predominates (Figure 4.5). For anti-candidal purposes, this combination should be ideally mixed in ratios of 6:4, 5:5, 4:6, 3:7, 2:8 and 1:9 (*L. angustifolia*: *C. sinensis*). Against the micro-organism *S. aureus*, synergy is observed regardless of the ratio mix. This outcome mirrors the expectations of layman literature indicating that in certain instances a specific ratio is not needed to bring about a desired therapeutic effect (Lawless, 1995; Shealy, 1998; Hili, 2001).



Figures 4.5 Isobologram representation of *Citrus sinesis* essential oil in combination with *L. angustifolia* essential oil at various combination ratios. ● Indicates *L. angustifolia* in majority; ●indicates *C. sinensis* in majority and ■indicates 1:1 ratio.

***D. carota* and *L. angustifolia* in combination:** is indicated in the treatment of bacterial and viral respiratory infections (Sellar, 1992). *L. angustifolia* has been indicated in the treatment of yeast and *Staphylococcal* related infections (Sellar, 1992; Curtis, 1996). No information could be attained to support the use of *D. carota* oil for these infections. When *L. angustifolia* was combined with *D. carota* against the micro-organism *C. albicans* in various ratios, eight of the ratios investigated demonstrated synergistic antimicrobial effects (Figure 4.6). One ratio (*L. angustifolia*: *D. carota*, 9:1) indicated an additive antimicrobial interaction. Additive antimicrobial interactions were also noted for all nine ratios against *S. aureus*. This combination should be ideally mixed in the ratio of (*L. angustifolia*: *D. carota*) 1:9 in order to obtain the more potent anti-staphyloccocal effect.

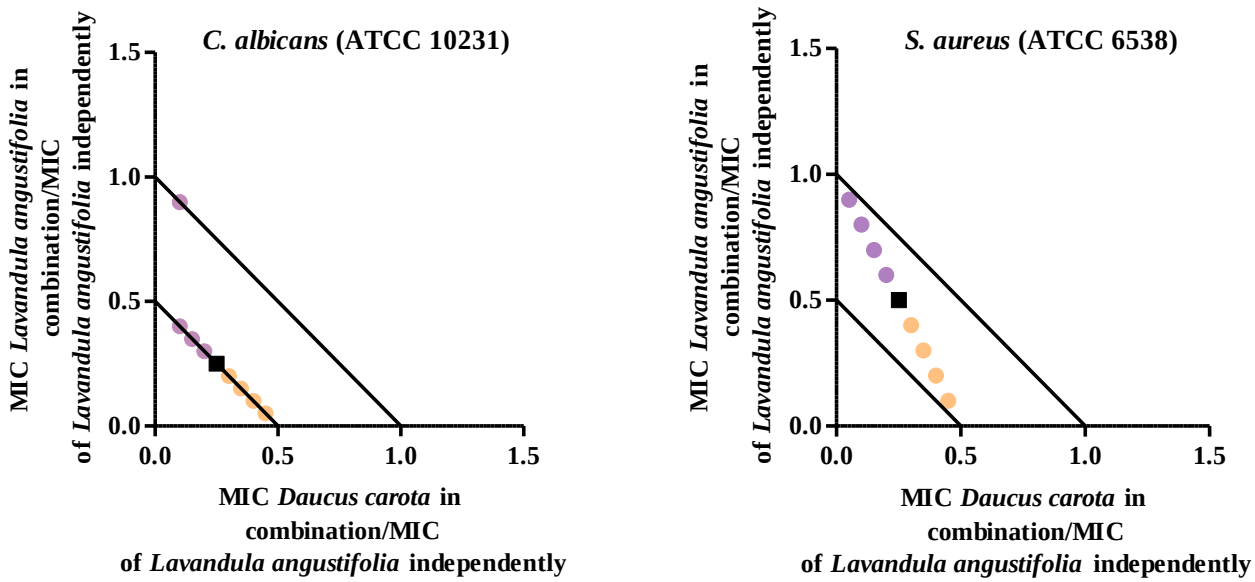


Figure 4.6 Isobologram representation of *Daucus carota* essential oil in combination with *L. angustifolia* essential oil at various combination ratios. ● Indicates *L. angustifolia* in majority; ● indicates *D. carota* in majority and ■ indicates 1:1 ratio.

***J. virginiana* and *L. angustifolia* in combination:** is indicated in the treatment of bacterial respiratory and yeast infections such as thrush (Lawless, 1995; Curtis, 1996). *L. angustifolia* in combination with *J. virginiana* against the micro-organism *C. albicans* in various ratios demonstrated a synergistic effect for all nine ratios investigated (Figure 4.7). Against the micro-organism *S. aureus*, a predominant additive antimicrobial effect was noted. This result is interesting as it validates the indication of these essential oils in combination for the treatment of thrush. In order to achieve the greatest anti-staphylococcal effect this combination should be placed in a ratio of (*L. angustifolia*: *J. virginiana*) 7:3. It should also be noted that when the concentration of *J. virginiana* to *L. angustifolia* essential oil reaches 9:1 the antimicrobial activity of the combination becomes non-interactive.

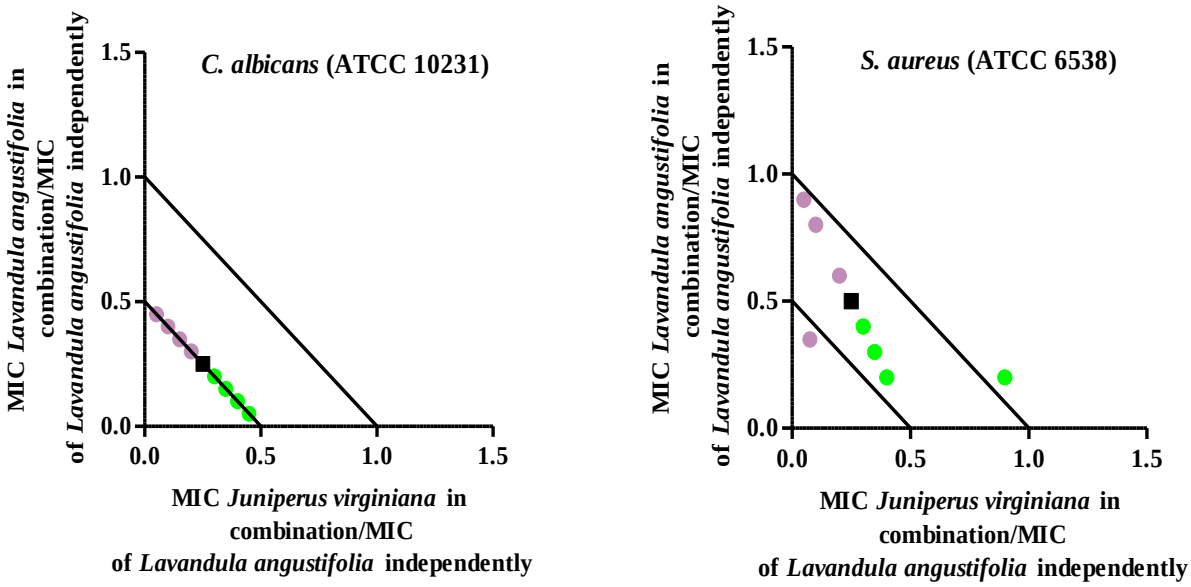


Figure 4.7 Isobologram representation of *Juniperus virginiana* essential oil in combination with *L. angustifolia* essential oil at various combination ratios. ● Indicates *L. angustifolia* in majority; ● indicates *J. virginiana* in majority and ■ indicates 1:1 ratio.

From the data obtained on the antimicrobial interactions of these four essential oil combinations in varying ratios, it is apparent that the concentration of the essential oils in the combinations is, in certain instances, pivotal in achieving a desired antimicrobial effect. Although no antagonism was noted for the combinations tested in varying ratios, it is evident that the possibility of poor antimicrobial efficacy and potential adverse effects are great when there is no specific indication for the mixing of essential oil combinations for a specific therapeutic purpose

4.3. Overview

With the modern age and development of technology, information and resources are available to us at the slide of a mouse and the touch of key. This easy access to information has created an opportunity for people to become more in control of their own health as increased numbers of individuals scour the internet for diagnosis to symptoms and home treatment options (White and Horvitz, 2009). Due to the ease of access and use of essential oils,

aromatherapy has become a popular means of home treatment for a diversity of conditions (Hili, 2001).

With this in mind, a number of layman resources have been published on the use of essential oils for antimicrobial purposes. Many of these pieces of literature indicate only which essential oils "blend" well for a particular therapeutic purpose but give no indication as to how they should be combined. This lack of direction causes the consumer to mix these essential oils in equal proportions due to assumption or varied ratios based on preference. Some studies have identified the combined antimicrobial effects of essential oils in equal proportions (Gutierrez *et al*., 2008; Škrinjar and Nemet, 2009) while others have investigated the effects of essential oils in varied ratios (Fu *et al*., 2007).

A study performed by Gutierrez *et al*. (2008) investigated the antimicrobial interaction of oregano essential oil in combination with basil, lemon balm, marjoram, rosemary, sage and thyme to determine possible synergistic interactions when tested against three bacterial strains (*B. cereus*, *E. coli* and *P. aeruginosa*). From the 1:1 analysis of these essential oils it was determined that a predominant additive interaction was determined against *B. cereus* while indifference was experienced against *E. coli* and *P. aeruginosa*.

Škrinjar and Nemet (2009) further sought to combine the essential oils of marjoram, thyme, basil, lemon balm, rosemary and sage in equal ratios in order to determine possible synergistic antimicrobial interactions when investigated against *L. monocytogenes*. From the ΣFIC analysis it was determined that the predominant antimicrobial interaction of the essential oils was indifference with the lowest ΣFIC value determined for the combination of marjoram and basil of 0.75.

Another study conducted by Fu *et al*. (2007) aimed to determine the antimicrobial interaction of clove and rosemary essential oil at equal and varying concentration ratios. At equal proportions a predominantly additive antimicrobial interaction was determined against the micro-organisms investigated with a non-interaction noted against *P. aeruginosa*. When placed in varying ratios no difference in antimicrobial interaction was determined for the majority of the micro-organisms studied. Synergy was however noted for the combinations 3:1 and 5:1 (clove: rosemary) against *C. albicans* and antagonism against *A. niger* for the

ratios 1:5, 1:7 and 1:9 (clove: rosemary). This study confirms that variances occur in antimicrobial interaction of essential oils when placed in different concentration ratios.

Due to the complexity of essential oil chemistry and the lack of information regarding the safety of these substances, it is of concern that these products are freely available and experimentation with their use broadly encouraged. Individual essential oils such as *M. alternifolia* have been proven to be toxic to animals and humans through a number of studies (Söderberg *et al*., 1996; Hayes *et al*., 1997; Darben *et al*., 1998; Bischoff and Guale, 1998; Hart *et al*., 2000; Mikus *et al*., 2000; Brand *et al*., 2001; Schnitzler *et al*., 2001), while according to studies such as Fu *et al*. (2007) varying concentrations of clove and rosemary cause antagonistic antimicrobial effects. This difference in therapeutic effect is most adequately seen in the plant product Digoxin, an antiarrythmic drug isolated from the plant foxglove. Digoxin has a very narrow therapeutic window and as such the therapeutic properties of this compound are greatly dependant on the concentration in which it is applied (Beers *et al*., 2006). The safety and antimicrobial potential of an essential oil or its major chemical constituent has been determined in this study as being concentration dependent. The belief that products obtained from nature are safer than conventional medicines is a dangerous misconception, as every piece of material on earth is constituted of matter, some harmless and some toxic. It is therefore important to understand the potential dangers afforded to the use of essential oils as well as their chemical compounds for therapeutic purposes in order to maintain patient safety.

These studies further augment the findings of this research in that although essential oils show some promise in antimicrobial activity alone or in combination, the potency of this activity is greatly affected by the concentrations at which the essential oils in the combinations are mixed. As aromatherapy is based on holistic treatment, the use of smell and self expression play an intricate role in its practice. As such, in some instances, no specific formula is given for essential oil combinations. However, although the practice of aromatherapy is based on a holistic approach to treatment, perhaps a more specific and scientific approach could be afforded to essential oil combinations intended solely for antimicrobial purposes in order to take advantage of the potency of particular ratios of combined essential oils.

4.4. General conclusion

- *Lavandula angustifolia* essential oil demonstrated a 90% noteworthy antimicrobial effect when tested against seven reference bacterial strains, three reference yeast strains and four clinical strains.

- *C. tropicalis* was the micro-organism most susceptible to the antimicrobial effects of *L. angustifolia* with an average MIC value of 0.75 mg/ml.

- The ΣFIC analysis of the essential oil combinations indicated that these oils possess favourable antimicrobial activity when placed in combination, as 23.5% of the combinations were synergistic, 52.3% additive, 23.5% non-interactive and 0.5% antagonistic.

- *Santalum album* was the essential oil with the best overall effect when combined with *L. angustifolia* in 1:1 combinations (MIC value of 0.92 mg/ml).

- *C. albicans* was the micro-organism most affected by the essential oils in variable ratios with the predominance of combinations demonstrating synergy against this micro-organism.

- When *L. angustifolia* was combined with a selection of oils at various ratios, it was generally found that the oil to which *L. angustifolia* is combined (i.e. *D. carota*, *J. virginiana* and *C. sinensis*) was responsible for a greater antimicrobial outcome.

- The combination of *L. angustifolia* and *C. sinensis* at varied ratios demonstrated the best overall antimicrobial effect with synergy identified against both micro-organisms tested.

- No antagonism was noted for any of the combinations tested in various ratio concentrations.

CHAPTER 5
THE ROLE OF MAJOR CONSTITUENTS IN THE ANTIMICROBIAL PROPERTIES OF *L. ANGUSTIFOLIA* ESSENTIAL OIL COMBINATIONS

5.1. Introduction

As discussed in Chapter 3 (essential oil constituent analysis), essential oils are products of a complex mixture of chemical compounds. Due to the complexity of the composition it is difficult to identify if a pharmacological action is dependent on a particular compound or a combination of compounds. Very often the effects of the essential oil is based on the relationship of the compounds within, as these chemical compounds have the ability to complement or inhibit the actions of another resulting in synergistic or antagonistic relationships. However, in some instances, single chemical compounds are responsible for the pharmacological action of an essential oil. Studying this particular compound for its action is known as the 'molecular approach' (Clarke, 2008).

Due to the complexity of essential oil chemistry, it is separated into certain classes based on molecular structure. In essential oil chemistry, each compound fits into two broad classes, namely the class of hydrocarbons or the class of oxygenated hydrocarbons. From these classes they are further separated into subdivisions such as terpenes and esters, dependant on their molecular structure. According to literature, antimicrobial activity is highest in essential oils greater in aldehyde and phenol concentrations, with weaker activity seen with compounds containing terpenes and alcohols (Bassolé and Juliani, 2012).

Of the essential oils selected for analysis, 12.3% are composed of either aldehyde or phenol compounds as major chemical constituents, of which 57.1% of these essential oils are primarily comprised of these compounds. Phenols are very reactive and are believed to demonstrate strong antiseptic, anti-infective and bactericidal properties as well as the ability to stimulate the immune system. Essential oils containing aldehydes are skin irritants due to the aldehydes reactivity to oxygen and are believed to be anti-infective in property (Clarke,

2008). From the essential oils selected for analysis the most commonly identified aldehydes were geranial and neral, while eugenol was the most commonly noted phenol.

Terpenes are liquids with a strong odour that evaporate quickly. This class of compounds is believed to demonstrate antiseptic, antiviral and bactericidal therapeutic properties. Alcohols are believed to be antiseptic, antiviral and antibacterial in action, with the ability to stimulate the immune system. Essential oils high in alcohol functional groups are considered safe for use due to their low level of toxicity (Clarke, 2008). Terpenes and alcohols are the major constituents in 87.7% of the essential oils selected for analysis, of which 54.0% percent of these essential oils are primarily comprised of these compounds. From the essential oils selected for analysis the most commonly identified terpene was limonene, while linalool was the most commonly noted phenol.

According to literature, essential oils predominant in ketones and esters are believed to be inactive (Bassolé and Juliani, 2012). Of the essential oils selected for analysis, 33.3% are composed of either ketone or ester compounds as major chemical constituents, of which 5.3% percent of these essential oils are primarily comprised of these compounds. Essential oils high in esters are considered safe for use due to their low level of toxicity and are considered primarily antifungal in activity. Ketones are very rarely found in essential oils and are considered hazardous for use (Clarke, 2008). From the essential oils selected for analysis the most commonly identified ketone was menthone, while linalyl acetate and bornyl acetate were the most commonly noted esters.

Many scientific studies have been undertaken to identify the antimicrobial effects of single essential oil chemical constituents (Didry *et al*., 1994; Carson and Riley, 1995; Hammer *et al*., 2003; Skaltsa *et al*., 2003; Terzi *et al*., 2007; van Vuuren and Viljoen, 2007; Loughlin *et al*., 2008; Shokeen *et al*., 2008; Hemaiswarya and Doble, 2009; Iten *et al*., 2009; Ahmad *et al*., 2010; Khan *et al*., 2010; Palaniappan and Holley, 2010; Qiu *et al*., 2010; Soković *et al*., 2010; Hussain *et al*., 2011; Lima *et al*., 2011; Moon *et al*., 2011; Njume *et al*., 2011; Paraschos *et al*., 2011; Zore *et al*., 2011; Castalho *et al*., 2012, Furneri *et al*., 2012) against a variety of micro-organisms. Other studies have aimed to identify the antimicrobial effects of single essential oil compounds in combination with common antimicrobial agents (Hemaiswarya and Doble, 2009; Ahmad *et al*., 2010; Moon *et al*., 2011, Zore *et al*., 2011),

while others have placed single essential oil compounds in combination in order to determine their combined effect (Didry *et al*., 1994; van Vuuren and Viljoen, 2007, Iten *et al*., 2009).

This chapter aims to apply the 'molecular approach' to determine if the major chemical compounds (Chapter 3) of the synergistic essential oil combinations identified in combination with *L. angustifolia* (Chapter 4) play a role in the antimicrobial effects observed for the essential oils.

5.2. Selection of constituents for combination studies

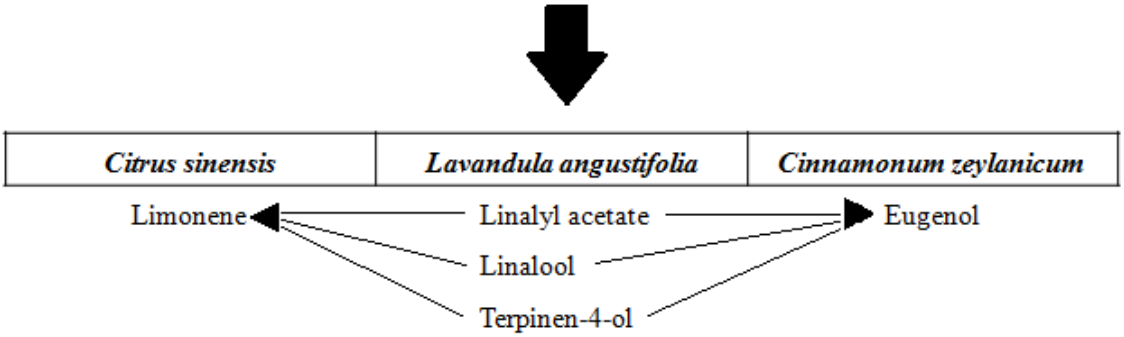
As the two essential oils demonstrating the most promising synergistic efficacy with *L. angustifolia* were *C. zeylanicum* and *C. sinensis* (Chapter 4 Section 4.2.4), their constituents were considered for further analysis to determine the interactive role when combined with major constituents from *L. angustifolia* (Table 5.1). The major chemical constituents were selected according to the determined chemotype and combined in various combinations with major compounds from *L. angustifolia* as indicated in Table 5.2.

Table 5.1 The percentage purity of the major chemical constituents selected for antimicrobial analysis.

Major chemical constituent	Percentage purity
(±)-Linalyl acetate (Fluka)	≥ 95%
(±)-Linalool (Fluka)	≥ 99%
(±)-Terpinen-4-ol (Fluka)	≥ 97%
Eugenol (Fluka)	≥ 98%
(+)-Limonene (Fluka)	> 95%

Table 5.2 A summary of the major chemical constituents investigated in combination to determine the role of chemistry in antimicrobial outcome.

Essential oil	Major chemical constituent/s	Percentage abundance
Lavandula angustifolia	Linalyl acetate	36.7
	Linalool	31.4
	Terpinen-4-ol	14.9
Cinnamonum zeylanicum	Eugenol	80.0
Citrus sinensis	Limonene	94.6



5.3. Results and Discussion

5.3.1. The antimicrobial effect of the major chemical constituents

Initially, the major chemical constituents of the two most promising essential oil combinations (*L. angustifolia* in combination with *C. zeylanicum* and *L. angustifolia* in combination with *C. sinensis*), as observed in the multiple ratio analysis (Chapter 4), were investigated independently to determine their antimicrobial effects (Table 5.3). Previous studies confirm antimicrobial efficacy of these major constituents against those micro-organisms investigated in this study (Table 5.3). Findings from this study demonstrate that *C. albicans* was the micro-organism most susceptible with MIC values ranging from 0.50 mg/ml (eugenol) to 2.00 mg/ml (limonene). Linalyl acetate, present in *L. angustifolia*, demonstrated the weakest antimicrobial effect against the micro-organism *S. aureus* with a mean MIC value of 4.00 mg/ml.

Table 5.3 The mean MIC values (n=3) for the major chemical constituents of *L. angustifolia*, *C. zeylanicum* and *C. sinesis.*

Essential oil	Major chemical constituent	Percentage abundance	MIC (mg/ml)		Previous research conducted
			C. albicans **(ATCC 10231)**	***S. aureus*** **(ATCC 6538)**	
L. angustifolia	Linalyl acetate	36.7	1.50	4.00	Hinou *et al*., 1989; Trombetta *et al*., 2005; van Zyl *et al*., 2006
	Linalool	31.4	1.00	1.00	Soković *et al*., 2010; Hussain *et al*., 2011; Paraschos *et al*., 2011
	Terpinen-4-ol	14.9	1.00	1.00	Carson and Riley, 1995
C. zeylanicum	Eugenol	80.0	0.50	1.00	Ahmad *et al*., 2010; Qui *et al*., 2010; Zore *et al*., 2011
C. sinensis	Limonene	94.6	2.00	2.00	van Vuuren and Viljoen, 2007; Soković *et al*., 2010
Positive control	Ciprofloxacin		NR*	$0.30x10^{-3}$	
	Amphotericin B		$0.60x10^{-3}$	NR	

NR*= Not relevant control for pathogen.

Many studies have been undertaken to determine the antimicrobial effect of eugenol against a wide variety of micro-organisms (Didry *et al*., 1994; Shokeen *et al.*, 2008; Hemaiswarya and Doble, 2009; Ahmad *et al*., 2010; Palaniappan and Holley, 2010; Qiu *et al.*, 2010; Moon *et al*., 2011 and Zore *et al.*, 2011), as well as its effect in combination with common antimicrobial agents (Hemaiswarya and Doble, 2009; Ahmad *et al.*, 2010; Moon *et al.*, 2011 and Zore *et al.*, 2011) and other major chemical constituents (Didry *et al.*, 1994). In a study conducted by Ahmad *et al*. (2010) eugenol was tested against 30 fluconazole-sensitive *C. albicans* strains and nine fluconazole-resistant strains and the MIC determined by the broth microdilution method. Eugenol obtained MIC values of 0.48-0.50 mg/ml against varied *C. albicans* strains. These results are congruent with those obtained in this study. Another study conducted by Zore *et al*. (2011) aimed to identify the anti-candidal effect of eugenol against 48 *C. albicans* strains by means of broth microdilution assays. Eugenol was identified as having inhibited 25 *C. albicans* strains. This outcome is congruent with that of the findings obtained in this study. A study conducted by Qiu *et al*. (2010) aimed to identify the antimicrobial effect of eugenol against 26 strains of *S. aureus*. From the study it was determined that eugenol demonstrated noteworthy antimicrobial effects with MIC values

ranging from 0.13-0.51 mg/ml. This outcome is more or less congruent with that in this study (Table 5.3) as eugenol demonstrated a mean MIC value of 1.00 mg/ml against *S. aureus*.

In order to determine possible antimicrobial interactions, these chemical constituents were further investigated in combination and the ΣFIC reported (Table 5.4).

5.3.2. The antimicrobial effect of the major chemical constituents of essential oils at a 1:1 ratio

When the three major chemical constituents found in *L. angustifolia* essential oil were placed in combination with the major chemical constituents of *C. zeylanicum* and *C. sinensis* at 1:1 ratios, the most promising synergistic interaction identified against both micro-organisms tested was for the combination of linalyl acetate with limonene, which demonstrated a ΣFIC of 0.30 (Table 5.4). According to literature no investigation has been undertaken to identify the antimicrobial effect of these chemical compounds in combination. However, a study has been conducted by van Vuuren *et al* (2007) in which the interactive properties of limonene were investigated in combination with 1, 8-cineole. When placed in equal ratios predominance in non-interactive antimicrobial effect was noted with antagonism demonstrated against *P. aeruginosa* (ΣFIC of 4.00), *Klebsiella pneumoniae* (ΣFIC of 4.00) and *Moraxella catarrhalis* (ΣFIC of 16.00). Limonene is a common chemical constituent in essential oils with 19.6% of the essential oils investigated in this study having limonene as a major chemical constituent (See Chapter 3, Table 3.1). On its own limonene has demonstrated noteworthy antimicrobial potential (Table 5.3). When placed in combination with other chemical compounds, this effect is potentiated two to four fold (Table 5.4). This result is interesting and may give some indication as to why the essential oils predominant in this major chemical constituent show strong antimicrobial interaction when placed in combination with *L. angustifolia*. Although limonene demonstrates noteworthy antimicrobial potential individually and at 1:1 ratios, it has been determined by studies such as van Vuuren *et al*. (2007) that this effect is greatly affected by concentration. When placed in nine varying ratios in combination with 1, 8-cineole, the combination demonstrates vast differences in antimicrobial effect. Therefore, in order to better understand the antimicrobial effects of the major chemical constituents in combination, these compounds were further investigated in varied ratios.

Table 5.4 Average MIC (mg/ml) and ΣFIC values for the major chemical constituents of *L. angustifolia*, *C. zeylanicum* and *C. sinesis.*

Major chemical constituent	*C. albicans* (ATCC 10231)		*S. aureus* (ATCC 6538)	
	MIC	ΣFIC	MIC	ΣFIC
Linalyl acetate* and eugenol†	0.50	0.67	1.00	0.63
Linalool* and eugenol	0.50	0.75	1.00	1.00
Terpinen-4-ol* and eugenol	0.50	0.75	1.00	1.00
Linalyl acetate and limonene‡	0.50	**0.30**	4.00	1.50
Linalool and limonene	2.00	1.50	1.00	0.75
Terpinen-4-ol and limonene	1.00	0.75	1.00	0.75

Bold indicates synergy, * indicates major chemical constituents of *L. angustifolia*, † indicates major chemical constituents of *C. zeylanicum* and ‡ indicates major chemical constituents of *C. sinensis*.

5.3.3. The antimicrobial effect of the major chemical constituents at various ratios

When the compounds were placed in 1:1 ratios, the concentration of the major chemical constituents did not reflect the exact concentration at which it would be found should the two oils be combined (Table 5.5). Therefore, in order to take this into consideration, varying ratios, including that which would reflect combined concentrations (□) as reflected in the combination of oils (equation 5.1) were considered and the results presented as isobolograms.

Equation 5.1:

Amount of constituent A in oil A (%) + Amount of constituent B in oil B (%) = AB

Percentage of A in AB = A(%)/AB = First half of ratio

Percentage of B in AB = B(%)/AB = Second half of ratio

Table 5.5 The estimated calculated ratio of the combined chemical constituents that best represents the relation of these compounds in the 1:1 combinations of the primary oils.

Major chemical constituents with percentage abundance				Estimated calculated ratio of combined constituents	Representative position on isobologram	
					C. albicans	*S. aureus*
Linaly acetate*	36.7	Eugenol†	80.0	3:7	A	G
Linalool*	31.4	Eugenol	80.0	3:7	B	H
Terpinen-4-ol*	14.9	Eugenol	80.0	2:8	C	I
Linaly acetate	36.7	Limonene‡	94.6	3:7	D	J
Linalool	31.4	Limonene	94.6	2:8	E	K
Terpinen-4-ol	14.9	Limonene	94.6	1:9	F	L

* indicates major chemical constituents of *L. angustifolia*, † indicates major chemical constituents of *C. zeylanicum* and ‡ indicates major chemical constituents of *C. sinensis*.

When the constituent combinations for *L. angustifolia* and *C. zeylanicum* were examined against *C. albicans* a predominantly additive antimicrobial effect was noted (Figure 5.1). Synergy was noted (three of the nine ratios investigated) in the combination where linalool (*L. angustifolia*) was combined with eugenol (*C. zeylanicum*). Against *S. aureus*, a notifiably additive antimicrobial effect was determined for all the combinations investigated (Figure 5.1). Synergy was noted for the combination of linalyl acetate and eugenol against *S. aureus* at the ratio where nine parts of linalyl acetate was combined with one part eugenol. No antagonism was noted for any of the combinations against both micro-organisms, while *C. albicans* was determined as being the most susceptible to the effects of these chemical constituent combinations. The ratios A-F (Figure 5.1) as reflected in the composition of primary essential oil blends demonstrate dominance in additive antimicrobial effects. This outcome was also noted when *L. angustifolia* and *C. zeylanicum* were combined.

In past studies, linalool has demonstrated some antimicrobial potential against a variety of micro-organisms. In a study conducted by Hussain *et al*. (2011) the antimicrobial effect of this major chemical constituent was investigated against four Gram-positive pathogens (*S. aureus, B. cereus, Bacillus subtillus* and *Bacillus pumulis*) and two Gram-negative (*E. coli* and *P. aeruginosa*) organisms. Average MIC values of 0.43 mg/ml and 0.95 mg/ml were determined for linalool against the Gram-positive and Gram-negative strains, respectively. Another study conducted by Paraschos *et al*. (2011) determined linalool to have poor antimicrobial potential individually against *E. coli* (MIC of 12.72 mg/ml), *S. aureus* (MIC of 14.28 mg/ml) and *C. albicans* (MIC of 9.88 mg/ml). The research conducted by Hussain *et*

al. (2011) augments the findings of this research determining the antimicrobial potential of this chemical compound individually. Linalool has also been investigated for its antimicrobial potential in combination with other chemical compounds in equalling ratios. In a study conducted by Bassolé *et al*. (2010) linalool was placed in combination with either carvacrol or eugenol at 1:1 ratios. Synergy was identified for these combinations against the pathogens *L. monocytogenes*, *E. coli*, *E. aerogenes* and *P. aeruginosa*. There are a very limited number of studies available on the antimicrobial activities of essential oil major chemical constituents in varying ratios, with this combination investigated for the first time by this means of analysis.

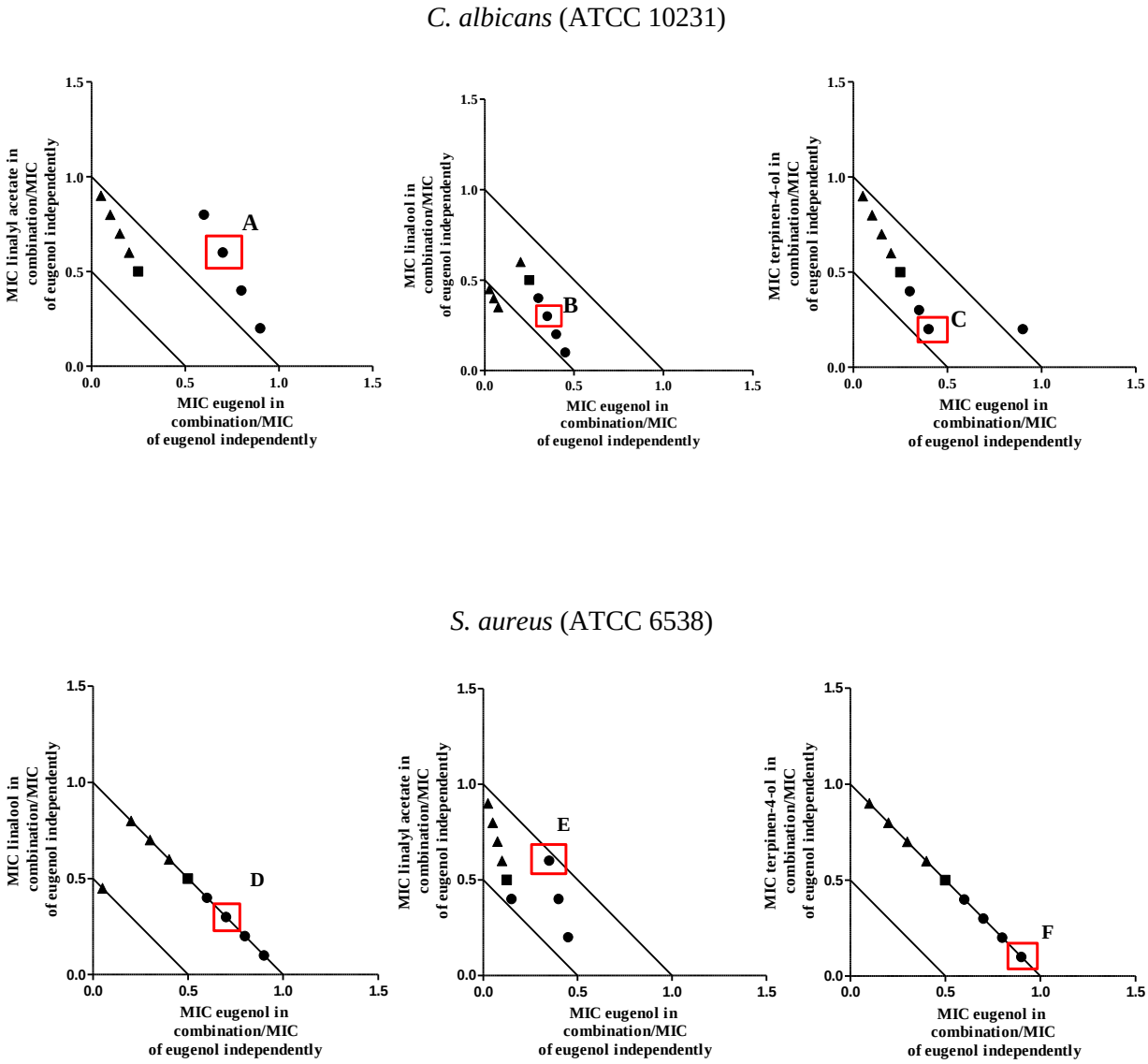


Figure 5.1 Isobologram representations of the major chemical constituents of *L. angustifolia* in combination with the major chemical constituent of *C. zeylaniucm* at various ratios.

▲ Indicates the major chemical constituents of *L. angustifolia* in majority; ● indicates the major chemical constituent of *C. zeylaniucm* in majority, ■ indicates 1:1 ratio; ☐ indicates ratio of constituents that would be found when two oils were combined.

When the constituent combinations for *L. angustifolia* and *C. sinensis* were examined against *C. albicans*, dominance in non-interactive antimicrobial effects were demonstrated (Figure 5.2). Synergy was noted for all nine ratios investigated when linalyl acetate (*L. angustifolia*) and limonene (*C. sinensis*), were combined. This combination, at 1:1 ratios using the ΣFIC analysis, also demonstrated the greatest synergistic effects. Against *S. aureus*, a predominantly additive antimicrobial effect was observed with non-interactive effects determined for the combination of linalyl acetate and limonene (Figure 5.2). No antagonism was noted for any of the combinations against both micro-organisms. The ratios G-L (Figure 5.2), as reflected in the composition of primary essential oil blends demonstrate predominantly non-interactive antimicrobial effects. This outcome is not noted for the primary oils as synergy was determined. This outcome further augments the principles often encounted by researchers (Marino *et al*., 2001; Delaquis *et al*., 2002; Burt, 2004; Koutsoudaki *et al*., 2005; Viljoen *et al*., 2006) that not only major compounds play a role in efficacy.

Limonene, as described earlier, has some antimicrobial potential when investigated individually (van Vuuren *et al*., 2007). Studies have been conducted on this chemical compounds antimicrobial potential when placed in 1:1 and varying ratios. In a study conducted by Tserennadmid *et al*. (2011), limonene was placed in combination with *α*-pinene and tested against *S. cerevisiae* at a 1:1 ratio. Synergy was identified for this combination. Besides this study, there is very limited information available on the antimicrobial activities of oil constituents in ratio combinations. A study conducted by van Vuuren *et al*. (2007), however placed the varying isomers of limonene in combination with 1, 8-cineole in nine different ratios to determine possible changes to the antimicrobial outcome of the combinations investigated. Against the micro-organism *S. aureus* the greatest variances were noted. When the combination of 1,8-cineole and (+)-limonene are placed in varying ratios against *S. aureus* and the antimicrobial potential determined, ratios higher in 1,8-cineole demonstrated additive antimicrobial interactions. Non-interactive profiles were found for ratios higher in (+)-limonene concentration. This result further validates the claim that the antimicrobial potential of combinations is dependent on the ratio at which the entities are

combined and in the case of the earlier study, even isomeric distribution of molecules may play a role. To note; in this study, the (+)-limonene was used, and the role of stereochemistry for molecules with chiral carbons needs to be further explored.

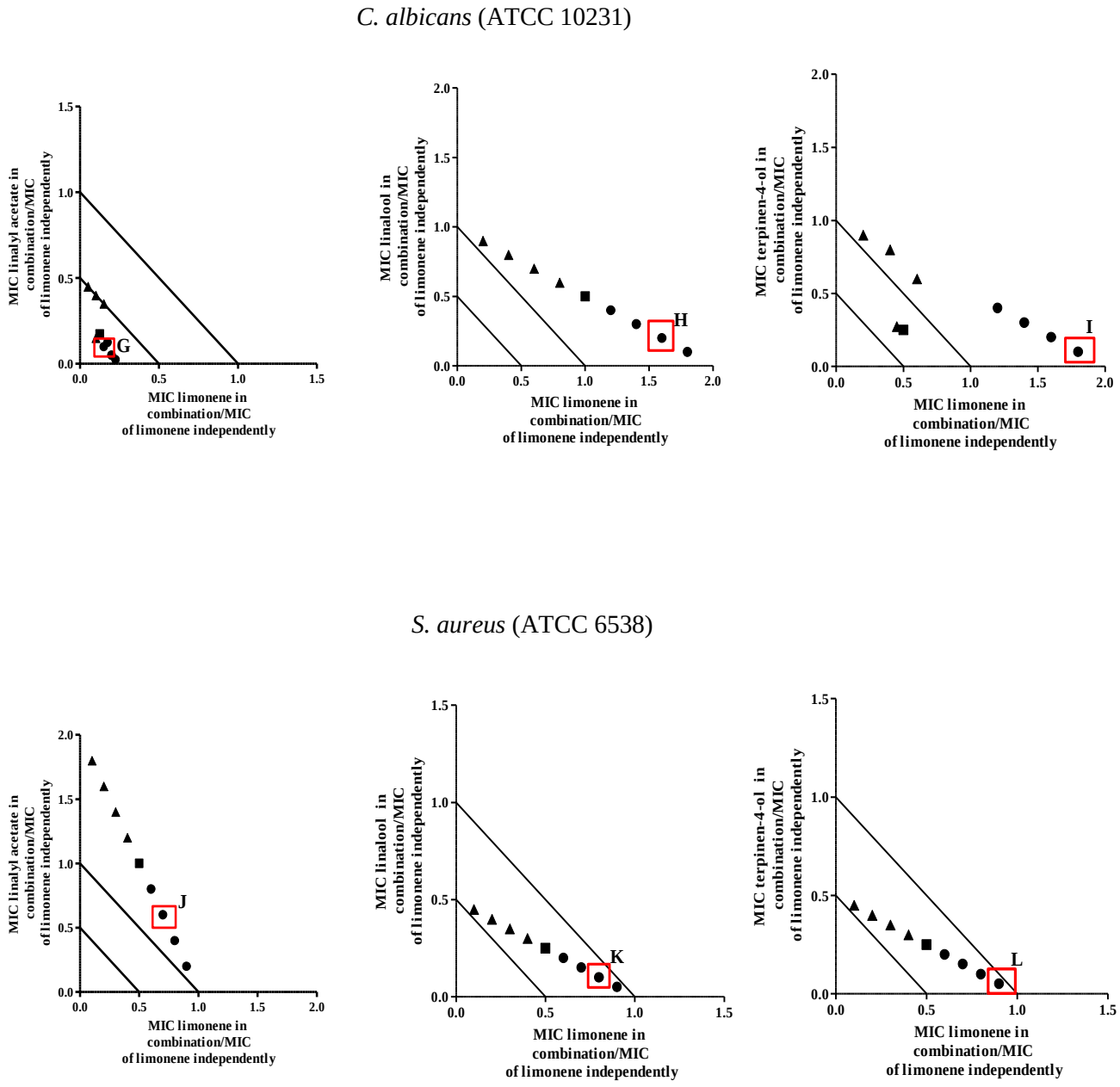


Figure 5.2 Isobologram representations of the major chemical constituents of *L. angustifolia* in combination with the major chemical constituent of *C. sinensis* at various ratios.

▲ Indicates the major chemical constituents of *L. angustifolia* in majority; ● indicates the major chemical constituent of *C. sinensis* in majority, ■ indicates 1:1 ratio; □ indicates ratio of constituents that would be found when two oils were combined.

5.4. Overview

When investigating the antimicrobial potential of the major chemical constituents, all compounds investigated demonstrated noteworthy antimicrobial potential, with exception of linalyl acetate against *S. aureus* (MIC of 4.00 mg/ml). When investigated further by means of ΣFIC analysis, additive antimicrobial interactions were apparent with synergy identified for the combination of linalyl acetate and limonene. The interactions of these chemical compounds showed further promise when placed in varying ratios; however the potential of these compounds was dependent on the ratio in which they were combined. This outcome supports the approach of investigating the antimicrobial potential of individual chemical compounds, but also demonstrates the importance of possible interactions of entities in essential oils.

The antimicrobial potential of these chemical constituents demonstrated greater antimicrobial potential than those of the oils from which they originated. The chemical constituent, eugenol, responsible for the greatest antimicrobial effect individually of the compounds tested is known to be toxic to the liver and an irritant to the skin and mucous membranes (Clarke, 2008). This high toxicity profile may in turn be responsible for the exceptional antimicrobial potential of the essential oil *C. zeylanicum*; however the interaction of the major and minor chemical constituents of this oil may deem this oil safer for use. Thus, although the major chemical constituents of an essential oil may be responsible for the antimicrobial effects of the oil, one must consider that it is likely that the antimicrobial effect, safety and physical properties of essential oils are due to the interactive effects of the compounds comprising it.

The importance of the interaction between major and minor chemical compounds can be demonstrated on a larger scale using the earth's atmosphere as an example. The atmospheric composition of the earth is comprised of nitrogen, oxygen, argon (major chemical constituents), carbon dioxide, neon, helium, methane, krypton, water vapour and hydrogen (minor chemical constituents) (Egger, 2003). The interaction of these compounds is pivotal to the survival of the earth. In instances where certain chemical entities in this composition are increased an imbalance of the atmospheric composition occurs, resulting in devastating effects such as noxious photochemical smog (increase in nitrogen oxides and organic compounds), acid rain (increase in nitrogen and sulphur oxides), ozone depletion (increase in

chloroflurocarbons) and greenhouse effects (increase in water vapour, carbon dioxide, methane and nitrous oxide) (Edlin and Golanty, 2010). Without the correct balance of the compounds in the atmosphere damage occurs to the health of the earth as well as those that inhabit it. Similarly, the balances between major and minor compounds in a whole essential oil play a role in the antimicrobial potential of an essential oil. In a study conducted by Inouye *et al*. (2001) it was determined that the antimicrobial potential of three popular essential oils (Lavender, Rosemary and Thyme) was attributed to the minor chemical constituents of the oils. A further study conducted by Viljoen *et al*. (2006) demonstrated that the interaction between the minor constituents of *Artemisia afra*, *α*-thujone and *β*-thujone, was responsible for the antimicrobial potential of the essential oil. Therefore, when evaluating the antimicrobial potential of essential oils, it is important to be cognisant of the potential interactions of major and minor constituents comprising them.

According to Clarke (2008), "The science that gives us the knowledge and understanding of chemistry can enhance the use of essential oils, especially when linked with the experience and intuition of the aromatherapist." Therefore, this chapter sought not only to augment the potential antimicrobial effects of the major chemical constituents of essential oils, but also to confirm the possibility of the importance of the interactions that occur between the chemical constituents that comprise these oils.

5.5. General conclusion

- Eugenol was found to have the greatest antimicrobial effect (*C. albicans* MIC of 0.50 mg/ml) and linalyl acetate was the compound with the poorest antimicrobial effect (*S. aureus* MIC of 4.00 mg/ml).

- At equal concentrations, linalyl acetate with limonene showed the greatest synergistic effect against the micro-organism *C. albicans* (ΣFIC of 0.30).

- When investigating the varied combinations of the constituents of *L. angustifolia* and *C. zeylanicum*, linalool with eugenol demonstrated the greatest antimicrobial effect.

- For the varying combinations of the chemical constituents of *L. angustifolia* and *C. sinensis*, linalyl acetate and limonene demonstrated the greatest antimicrobial effect.

- No antagonism was noted for any of the combinations investigated in 1:1 and varying ratios.

CHAPTER 6
THE INTERACTION OF *L. ANGUSTIFOLIA* ESSENTIAL OIL IN COMBINATION WITH CONVENTIONAL ANTIMICROBIAL AGENTS

6.1. Introduction

6.1.1. Antimicrobial resistance

Penicillin, one of the earliest known antibiotics for clinical use, was used for the first time on February 12, 1941. Fletcher (1984) describes the anxiety of the professionals involved to discover and utilise a drug that would treat the growing cases of sepsis developing at the time. It is described how penicillin was applied clinically for the first time to a patient suffering from a combined *Staphylococcal* and *Streptococcal* septicaemia, originating from a sore on the lip. The antibiotic was applied via intravenous administration at an initial dose of 200 mg followed by 300 mg every three hours. In a period of five days the patient had miraculously begun to recover with obvious resolution of abscesses to the face. Unfortunately, during this period the supply of such an antibiotic was scarce and the patient died a month later due to lack of further treatment (Fletcher, 1984). A short period following the discovery of the clinical use of penicillin, resistant strains of *Staphylococci* were identified (Abraham and Chaine, 1940). Due to the developing cases in resistance, 80% of all patients diagnosed with *Staphylococcal* related infections in American hospital settings in 1955, succumbed to their illnesses (Fisher, 1994).

In recent years, antimicrobial resistance has been on the rise with an unknown number of resistant bacterial strains. As of 2009, the micro-organism *Enterococcus faecium*, responsible for endocarditis, cellulitis and bacteremia infections (Beers *et al*., 2006) had developed a high level of resistance to aminoglycoside antibiotics with no known alternative for treatment (Arias and Murray, 2009). Due to this growing level of resistance, there has been an increased need to develop and research new products with antimicrobial effects for use independently and in combination with current antibiotics, with researchers looking towards natural products and plants for solutions (Othman *et al*., 2011). In recent years, natural products such as essential oils and plant extracts have been used to treat common infectious ailments, with some hospitals, using essential oils in place of general antiseptics (Buckle, 2003).

With the prevention of emerging antimicrobial resistence in mind, a study was designed to determine if the popular lavender oil would demonstrate favourable interactions when combined with conventional antimicrobials. Many studies have been conducted on the effects of essential oils in combination with antibiotics (Avenirova *et al.*, 1975; Hemaiswarya *et al.*, 2008; Lorenzi *et al.*, 2009; Fadli *et al.*, 2011), as well as essential oils in combination with antifungal agents (Suresh *et al.*, 1997; Shiota *et al.*, 2000; Shin and Kang, 2003; Giordani *et al.*, 2004), however, to the best of my knowledge, no studies have been conducted on the effects of *L. angustifolia* essential oil in combination with antimicrobial agents.

This chapter aims to identify the antimicrobial properties of *L. angustifolia* essential oil in combination with commercially available antimicrobial agents in order to determine their interactive profiles.

6.2. Results and Discussion

6.2.1. The antimicrobial effect of selected common antimicrobial agents

The four antimicrobial agents were first investigated for their antimicrobial efficacy against three test micro-organisms (Table 6.1). The MIC values determined were congruent with breakpoint recommendations (Chapter 2, Section 2.5.2.1, Table 2.3).

Table 6.1 Mean MIC values (n= 3) for the antimicrobial agents selected for combination analysis.

Antimicrobial agent	**MIC (µg/ml)**		
	C. albicans **(ATCC 10231)**	***S. aureus*** **(ATCC 6538)**	***P. aeruginosa*** **(ATCC 27858)**
Chloramphenicol	0.63	0.31	0.31
Ciprofloxacin	NA	0.11	0.04
Fusidic acid	NA	0.63	0.31
Nystatin	0.16	NA	NA

* NA = not applicable.

Chloramphenicol is a bacteriostatic antimicrobial agent found to bind to the 50S ribosomal subunit resulting in inhibition of protein synthesis of bacteria. According to Beers *et al.* (2006), chloramphenicol is specified in the treatment of *Rickettsia, Mycoplasma* and *Chlamydial* type infections however, studies have indicated chloramphenicol as having antifungal activity against *C. albicans* (Logemann *et al.*, 1961).

Ciprofloxacin is a first generation fluoroquinolone antibiotic which causes inhibition of the enzyme DNA gyrase (also known as topoisomerase II) which is responsible for cell replication. Ciprofloxacin is indicated in the treatment of many bacterial infections including *P. aeruginosa* and *S. aureus* related infections. Due to overuse of this class of antibiotic, antimicrobial resistance has developed (Beers *et al*., 2006).

Fusidic acid is a bactericidal antimicrobial agent that inhibits protein synthesis by interfering with elongation factors. It is specified in the treatment of topical, Gram-positive bacteria related infections such as *S. aureus* (MIMS Medical Desk Reference, 2004).

Nystatin belongs to the class of polyene antifungal agents and is specified in the treatment of oral and skin *C. albicans* infections. Nystatin acts by binding to ergosterol resulting in disruption to the fungal membrane (Beers *et al*., 2006).

6.2.2. The antimicrobial effect of *L. angustifolia* oil in combination with common antimicrobial agents

L. angustifolia essential oil was placed in combination with four antimicrobial agents and tested against three test pathogens to determine efficacy (Table 6.2). *L. angustifolia* in combination with nystatin demonstrated the best antimicrobial effect against the micro-organism *C. albicans* with an average MIC value of 1.00 mg/ml. Against both bacterial species, *S. aureus* and *P. aeruginosa*, *L. angustifolia* in combination with ciprofloxacin obtained the best MIC value of the combinations tested with an MIC of 0.25 mg/ml. This combination also demonstrated the best overall result against all the micro-organisms tested with an average MIC value of 0.25 mg/ml. Synergistic intractions were evident for *S. aureus* and *P. aeruginosa*, with ΣFIC values of 0.49 (*L. angustifolia* in combination with ciprofloxacin) and 0.29 (*L. angustifolia* in combination with chloramphenicol), respectively. No synergy was noted against the micro-organism *C. albicans*.

Table 6.2 Mean MIC values (n= 3) for *L. angustifolia* in combination with common antimicrobial agents.

Substance	Mean MIC (mg/ml) and ΣFIC					
	***C. albicans* (ATCC 10231)**		***S. aureus* (ATCC 6538)**		***P. aeruginosa* (ATCC 27858)**	
	MIC	**ΣFIC**	**MIC**	**ΣFIC**	**MIC**	**ΣFIC**
L. angustifolia + chloramphenicol	2.00	1.00	1.00	1.00	0.50	**0.29**
L. angustifolia + ciprofloxacin	*NA	NA	0.25	**0.49**	0.25	0.74
L. angustifolia + fusidic acid	NA	NA	0.75	0.56	0.50	1.13
L. angustifolia + nystatin	1.00	2.25	NA	NA	NA	NA

Bold = synergy, * NA = not applicable.

The combination with the lowest ΣFIC value, thus the greatest synergistic interaction, was observed for *L. angustifolia* in combination with chloramphenicol against *P. aeruginosa* (ΣFIC of 0.29). Even though no previous studies have been conducted on the antimicrobial interaction between *L. angustifolia* and chloramphenicol, chloramphenicol has been placed in combination with other natural therapies against different classes of micro-organisms, yielding positive results. In a study conducted by Duarte *et al.* (2012) chloramphenicol was placed in combination with *Coriandrum sativum* essential oil and the interaction determined against *Acinetobacter baumannii* by means of ΣFIC analysis. It was determined that this combination demonstrated considerable synergistic antimicrobial potential with ΣFIC values of 0.28. Another study conducted by Halawani (2009) determined the interactive properties of chloramphenicol and the major chemical constituents of the essential oil *Nigella sativa*, thymoquinone and thymohydroquinone, by means of ΣFIC analysis. From this study it was determined that chloramphenicol in combination with thymoquinone demonstrates synergistic antimicrobial effects against the micro-organisms *E. coli*, *S. aureus* and *S. typhimurium.* In combination with thymohydroquinone, synergy was determined against *S. aureus* and *P. aeruginosa*.

According to Hare (1960), the potentiation of an antimicrobial agent is a phenomenon that occurs when a technique is applied that results in the increased availability of the said antimicrobial agent in a test species for therapeutic purposes. In other words, potentiation of an antimicrobial agent results in a greater antimicrobial effect at a lower concentration. Many studies have been conducted on the potential of plant products for the potentiation of antimicrobial agents in the field of microbiology (Zhao *et al*., 2001; Hu *et al*., 2002; Gibbons *et al*., 2003; Gibbons *et al*., 2004; Oluwatuyi *et al*., 2004; Stapleton *et al*., 2004; Shibata *et*

al., 2005; Marquez *et al*., 2005; Al-Hebshi *et al*., 2006; Smith *et al*., 2007). A study conducted by Oluwatuyi *et al*. (2004) determined the plant extract of *Rosmarinus officinalis* to potentiate the antimicrobial activity of erythromycin by 16-32 fold against *S. aureus*; while Coutinho *et al*. (2009) determined the potentiation potential of *Turnera ulmifolia* against MRSA. These studies indicate the potential use of these compounds in combination with conventional antimicrobial agents for a greater therapeutic effect. From Table 6.3 it is noted that *L. angustifolia* causes potentiation of 50% of the antimicrobial agents to which it was combined. The most promising combination was noted for *L. angustifolia* with fusidic acid against *S. aureus* in which the antimicrobial effect of this agent was potentiated 5-fold (MIC of 0.63 μg/ml for fusidic acid individually and MIC of 0.12 μg/ml for the combination). Fusidic acid, as previously stated, is predominantly prescribed in the treatment of topical *Staphylococcal* infections, while *L. angustifolia* has also been deemed to be specified for the treatment of such infections. This potentiation of this antimicrobial agent suggests promise for the use of this combination in future pharmaceutical preparations.

Table 6.3 The antimicrobial effect of the individual components in the essential oil: antimicrobial combination.

Micro-organism	**MIC individual**			**MIC combination**		**ΣFIC**
	***LA* (mg/ml)**	**Antimicrobial (μg/ml)**				
C. albicans (ATCC 10231)	3.00	CH	0.63	2.00	0.63	0.83
		N	0.16	1.00	0.31	2.17
S. aureus (ATCC 6538)	2.00	CH	0.31	1.00	0.31	0.75
		C	0.11	0.25	0.08	0.38
		FA	0.63	0.75	0.12	0.43
P. aeruginosa (ATCC 27858)	2.00	CH	0.31	0.50	0.16	0.29
		C	0.04	0.25	0.08	0.74
		FA	0.31	0.50	0.16	1.13

LA indicates *L. angustifolia*, CH indicates Chloramphenicol, N indicates Nystatin, C indicates Ciprofloxacin, FA indicates Fusidic Acid.

Although the MIC and ΣFIC values obtained for these combinations indicate promising antimicrobial interactions, a further investigation was undertaken by means of variable ratio analysis in order to determine what interactions could be apparent when concentration ratios were altered.

6.2.3. The antimicrobial effect of *L. angustifolia* essential oil in combination with common antimicrobial agents at various ratios

L. angustifolia when combined with chloramphenicol in various ratios displayed the greater synergistic effect of the combinations tested against the micro-organism *C. albicans* (Figure 6.1a). When combined with nystatin in various ratios, a predominant non-interactive effect was noted (Figure 6.1b). Antagonism was observed for two ratios (*L. angustifolia*: nystatin, 8:2 and 7:3). It should be noted, that negative antimicrobial interactions of this combination occured where ratios are higher in *L. angustifolia*, therefore caution should be warranted when combining these agents. This adverse interaction of conventional medicine and natural therapies is not uncommon (van Vuuren *et al.*, 2008). Researchers suggest that when investigating the interaction between these forms of therapy, emphasis should not only be placed on identifying synergy, but antagonism as well, due to the likelihood of adverse drug interactions (Cuzzolin *et al*., 2006; van Vuuren *et al.*, 2008).

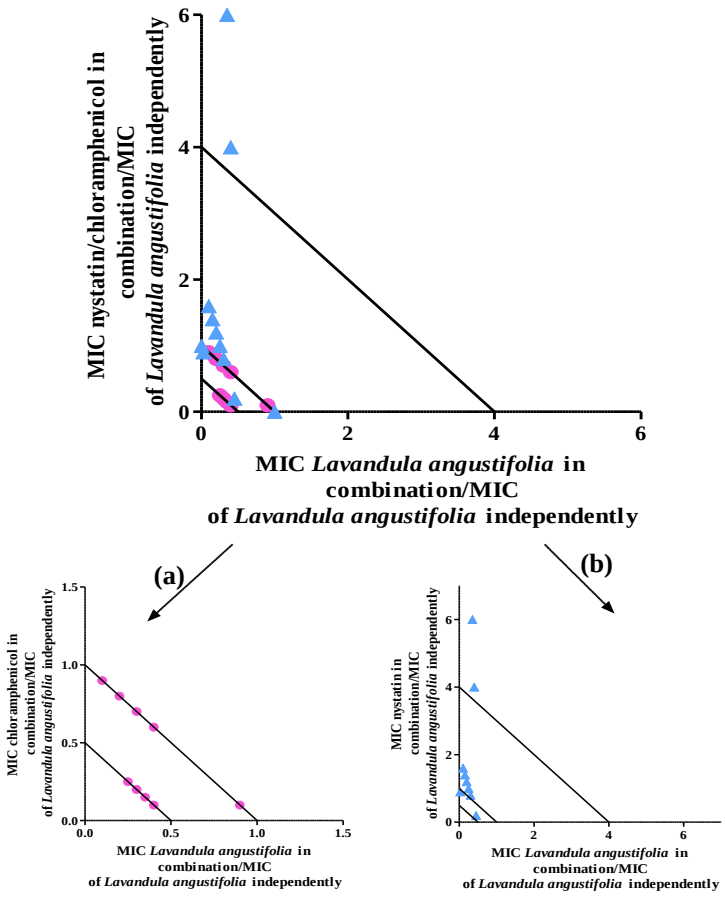


Figure 6.1 Isobologram representation of *L. angustifolia* essential oil in combination with chloramphenicol ● and nystatin ▲ at various ratios against *C. albicans* (ATCC 10231).

Overall, the interactions between *L. angustifolia* and the antimicrobial agents tested against *C. albicans* demonstrated a predominant non-interaction. This suggests that the therapeutic benefit of combining these agents in the future is limited to specific concentration ratios.

L. angustifolia when combined with chloramphenicol, ciprofloxacin and fusidic acid demonstrated synergistic and additive interactions against the micro-organism *S. aureus* (Figure 6.2). *L. angustifolia* in combination with fusidic acid (Figure 6.2a) demonstrated predominance in additive antimicrobial effects, with three ratios noted as synergistic (*L. angustifolia*: fusidic acid, 9:1, 8:2, and 7:3). When *L. angustifolia* was investigated in combination with ciprofloxacin in various ratios (Figure 6.2b), mainly synergistic interactions were noted. *L. angustifolia* when combined with chloramphenicol in various ratios (Figure 6.2c) was predominantly additive with synergy observed for four of the ratios investigated (*L. angustifolia*: chloramphenicol, 9:1, 8:2, 7:3, 6:4).

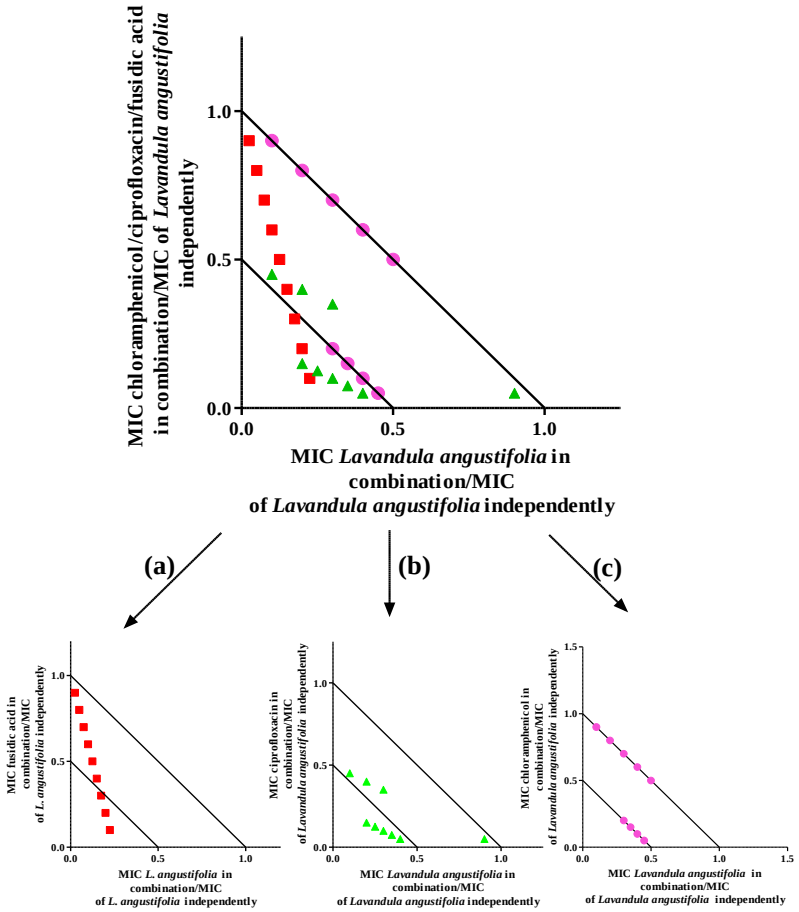


Figure 6.2 Isobologram representation of *L. angustifolia* essential oil in combination with chloramphenicol ●, ciprofloxacin ▲ and fusidic acid ■ at various ratios against *S. aureus* (ATCC 6538).

Staphylococcal infections are quite common and are considered the most pathogenic, due to the ability to develop resistance against commonly used antibiotics. These infections can be caused directly and present as pustules, abscesses or cellulitus (Beers *et al*., 2006). Interestingly, *L. angustifolia* essential oil is predominantly suggested for the treatment of topical superficial infections, as outlined in Chapter 1 Section 1.7, with conditions originating from *S. aureus* infections. Fusidic acid is also indicated primarily for the treatment of *S. aureus* infections, while chloramphenicol is prescribed in serious Gram-negative infections. Interestingly, for all the combinations investigated, ratios higher in concentrations of *L. angustifolia* oil result in a more favourable antimicrobial effect.

The overall antimicrobial effect for these combinations against *S. aureus* was additive with some synergistic interactions noted. Therefore the combination of *L. angustifolia* with these antimicrobial agents looks promising and may have a therapeutic advantage.

When *L. angustifolia* was combined with chloramphenicol, ciprofloxacin and fusidic acid in various ratios and tested against the micro-organism *P. aeruginosa* the overall interactions noted were additive (Figure 6.3). When *L. angustifolia* was combined with fusidic acid in various ratios (Figure 6.3a) it was noted that two ratios (*L. angustifolia*: fusidic acid, 9:1 and 5:5) demonstrating non-interactive properties. One combination was noted as synergistic (*L. angustifolia*: fusidic acid, 8:2). A similar effect was noted for the combination of *L. angustifolia* with ciprofloxacin (Figure 6.3b) where six of the nine ratios investigated demonstrated additive antimicrobial effects. When *L. angustifolia* was investigated in combination with chloramphenicol in various ratios (Figure 6.3c), mainly synergistic interactions were observed. Interestingly, for all the combinations investigated ratios higher in concentrations of *L. angustifolia* oil result in a less favourable antimicrobial effect.This is the opposite as what was observed with the Gram-positive *S. aureus* strain.

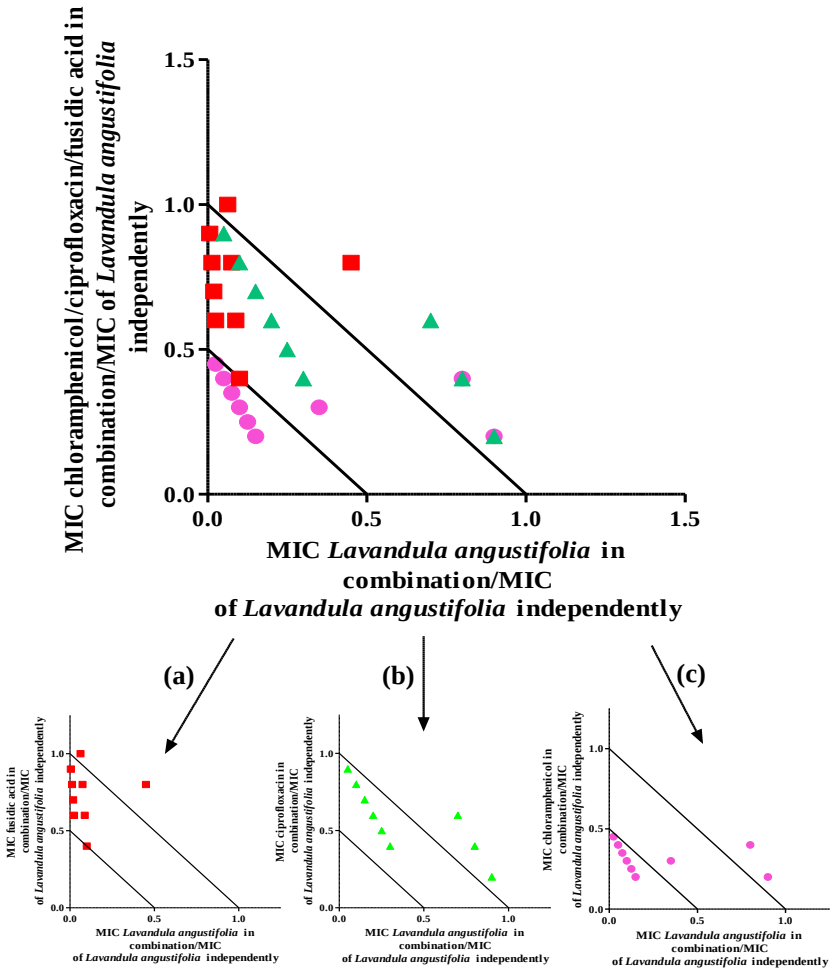


Figure 6.3 Isobologram representation of *L. angustifolia* essential oil in combination with chloramphenicol ● , ciprofloxacin ▲ and fusidic acid ■ at various ratios against *P. aeruginosa* (ATCC 27858).

6.3. Overview

According to Casal *et al*. (2005), presumably 90-95% of *S. aureus* strains are resistant to conventional antimicrobial agents. Furthermore, micro-organisms are developing multi-drug resistance resulting in fewer and fewer antimicrobial agents available for the treatment of infectious diseases (Hemaiswarya *et al*., 2008). In addition to the development of resistance, conventional antimicrobial agents are also accompanied by a large number of adverse effects and toxic drug-drug interactions (Rosato *et al*., 2007). Due to the surge in antimicrobial resistance and accompanying side effects, conventional medicine is desperately searching for newer antimicrobial agents to relieve the burden. Plant products have drawn the greatest level

of attention by scientists as a means of filling the void and alleviating the burden due to their proven *in vitro* antimicrobial potential. Researchers have moved towards identifying possible synergistic interactions between conventional antimicrobial agents and naturally occurring substances such as essential oils, in order to determine if an increase in antimicrobial effectiveness can be achieved (Harris, 2002; Rosato *et al*., 2007).

Many antimicrobial studies have been conducted to determine the effectiveness of plant essential oils in combination with conventional antimicrobial agents (Shin, 2003; Giordani *et al*., 2004; Rosato *et al*., 2007; Si *et al*., 2008; van Vuuren *et al*., 2008; Rosato *et al*., 2009; Mahboubi and Ghazian, 2010; Saad *et al*., 2010; Fadli *et al*., 2011; Malik *et al*., 2011; Moon *et al*., 2011; Duarte *et al*., 2012). A study conducted by Rosato *et al.* (2007) aimed to identify the interaction of *Pelargonium graveolens* in combination with norfloxacin, a second generation fluoroquinolone antibiotic. It was identified that the combination demonstrated synergistic activity against the micro-organism *B. cereus* and two strains of *S. aureus* with ΣFIC values of 0.50. A later study conducted by Rosato *et al*. (2009) was identified in which the study aimed to determine the antimicrobial interaction of *Origanum vulgare* and *P. graveolens* in combination with the antifungal agent, nystatin, against *C. albicans.* The study determined that *O. vulgare* obtained a synergistic interaction with nystatin with an ΣFIC value of between 0.11 and 0.17, while *P. graveolens* in combination with nystatin showed additive effects. Another study conducted by van Vuuren *et al*. (2008) aimed to identify the antimicrobial interaction of four essential oils (*M. alternifolia*, *T. vulgaris*, *M. piperita* and *R. officinalis*) in combination with two antimicrobial agents (ciprofloxacin and amphotericin B) against three micro-organisms (*S. aureus*, *K. pneumoniae* and *C. albicans*). *R. officinalis* in combination with ciprofloxacin against *K. pneumoniae* was the only combination to demonstrate synergy with ΣFIC values of 0.28 to 0.32. Many other antimicrobial studies have been conducted on the effects of essential oils in combination with antimicrobial agents and are further highlighted in Table 6.4.

Table 6.4 Previous antimicrobial studies conducted on the effects of essential oils in combination with conventional antimicrobial agents.

Essential oil/s	Antimicrobial agent/s	Bioactivity	Best combination	Best activity	Reference
Pelargonium graveolens	ketoconazole, amphotericin B	antifungal	*P. graveolens* + amphotericin B	*Aspergillus flavus* - ΣFIC of 0.63	Shin, 2003
Thymus vulgaris	amphotericin B	antifungal	*T. vulgaris* + amphotericin B	*Candida albicans* – MIC of 0.35 μg/ml	Giordani *et al*., 2004
Origanum vulgare	sarafloxacin, levofloxacin, polymycin, lincomycin, amoxicillin, ceftiofur, ceftriaxone, maquidox, florfenicol, doxycycline, kanamycin	antibacterial	*O. vulgare* + sarafloxacin, *O. vulgare* + florfenciol, *O. vulgare* + doxycycline	*Escherichia coli* - ΣFIC of 0.38	Si *et al*., 2008
Myrtus communis	amphotericin B	antifungal	*M. communis* + amphotericin B	*Aspergillis niger* - ΣFIC of 0.26	Mahboubi and Ghazian, 2010
Thymus maroccanus, Thymus broussonetii	amphotericin B, fluconazole	antifungal	*T. maroccanus* + fluconazole	*C. albicans* - ΣFIC of 0.27	Saad *et al*., 2010
T.maroccanus T.broussonetii	chloramphenicol	antibacterial	*T. maroccanus* + chloramphenicol	*E. coli* – MIC of 1.00 mg/ml	Fadli *et al*., 2011
P. graveolens	ciprofloxacin	antibacterial	*P. graveolens* + ciprofloxacin	*S. aureus* and *K. pneumoniae* - ΣFIC of 0.38	Malik *et al*., 2011
Syzygium aromaticum	ampicillin, gentamicin	antibacterial	*S. aromaticum* + ampicillin	*Streptococcus mutans, Streptococcus sobrinus, Streptococcus gordonii* - ΣFIC of 0.38	Moon *et al*., 2011
			S. aromaticum + gentamicin	*Streptococcus sanguinis, S. sobrinus, Streptococcus criceti, Porphyromonas gingivalis* - ΣFIC of 0.38	Moon *et al*., 2011
Coriandrum sativum	tetracycline, cefoperazone, chloramphenicol, ciprofloxacin, gentamicin, piperacillin	antibacterial	*C. sativum* + tetracycline	*Acinetobacter baumannii* - ΣFIC of 0.19	Duarte *et al*., 2012

In spite of the numerous publications supporting the use of essential oils in combination with conventional antimicrobial agents, caution should still be warranted as not all combinations have been proven to be synergistic. In a study conducted by van Vuuren *et al*. (2008) the combination of *M. alternifolia* essential oil and ciprofloxacin against the micro-organism *S. aureus* yielded antagonistic interactions, with ΣFIC values ranging from 5.17 to 7.70 for certain ratios. This negative outcome further highlights the need to investigate combinations at varying ratios to formulate optimal interactions. In so doing, we will effectively be able to formulate antimicrobial combinations involving essential oils and antimicrobial agents that result in fewer side effects while combating the surge in antimicrobial resistance.The safety of combining herbal or complementary therapy and conventional prescription medications is of a concern due to the high level of possible adverse herb-drug interactions. According to van Vuuren *et al*. (2008), patients often make use of essential oils as adjunct to conventional medicine for the treatment of certain symptoms associated with disease states such as HIV. Another study conducted by Bush *et al*. (2007) aimed to identify the number of patients that use complementary and alternative therapy as adjuncts to conventional medicine. From the 122 patients identified, 40% presented with combinations that had potential adverse interactions. The availability of natural products such as essential oils and the proposed benefit of the use of these compounds make patients vulnerable to these types of interactions. Therefore, not only is further research required into the possible potential of essential oils to relieve the antimicrobial resistance burden, but also to determine the safety of these products when used alongside prescription medication.

6.4. General conclusion

- *L. angustifolia* in combination with nystatin demonstrated the best antimicrobial effect against the micro-organism *C. albicans* with an ΣFIC value of 0.83.

- Synergy was determined for two of the combinations tested, *L. angustifolia* in combination with ciprofloxacin against *S. aureus* (ΣFIC of 0.49) and *L. angustifolia* in combination with chloramphenicol *against P. aeruginosa* (ΣFIC of 0.29).

- No antagonism was noted for the combinations investigated by means of ΣFIC calculation.

- Potentiation was noted for 50% of the antimicrobial agents when combined with *L. angustifolia* in 1:1 ratios.

- From the isobolograms it was identified that *L. angustifolia* provided the pivotal role in the combinations antimicrobial effect against the micro-organisms *C. albicans* and *S. aureus*, with ratios higher in *L. angustifolia* essential oil concentration showing considerably better antimicrobial effects.

- The combination of *L. angustifolia* essential oil and antimicrobial agent against the micro-organism *P. aeruginosa* demonstrated that ratios greater in antibiotic concentration demonstrating stronger antimicrobial effects.

- Against *C. albicans*, the combination of *L. angustifolia* and chloramphenicol was the most antimicrobially effective of the combinations investigated as four ratios demonstrated synergy.

- Synergy was identified for all the combinations investigated against the micro-organism *S. aureus* with the greatest level of synergy identified for the combination of *L. angutsifolia* and ciprofloxacin (five synergistic ratios).

- Against the micro-organism *P. aeruginosa*, the combination of *L. angustifolia* and chloramphenicol was once again the most synergistic of the combinations investigated with six synergistic ratios identified.

CHAPTER 7
DESIGN OF EXPERIMENTS

7.1. Introduction

In previous chapters, the aim was to determine if *L. angustifolia* in combination with other essential oils demonstrates antimicrobial activity when used independently and in combination. This was undertaken in consideration with the practice of aromatherapy and use as cited in literature (Sellar, 1992; Lawless, 1995; Curtis, 1996; Shealy, 1998; Hili, 2001; Buckle, 2003; Lawrence, 2005). These chapters have given some insight into the antimicrobial effects of essential oils combinations where two oils have been blended. It is important however, to take cognisance that in many practical applications, blends of more than two essential oils are often used.

While examining the literature for the aroma-therapeutic uses of *L. angustifolia*, it was observed that combinations including *L. angustifolia* often incorporated more than two essential oils. Some of these multiple combinations and the specificity of use are indicated in Table 7.1.

Table 7.1 Essential oil combinations incorporating *L. angustifolia* in blends with more than two other essential oils.

	Essential oil combination*	**Indication for use**	**Micro-organism responsible for infection**
1.	*Lavandula angustifolia* *Pelargonium odoratissimum* *Thymus vulgaris* *Eucalyptus globulus*	Varicose ulcers	*Streptococcal* and *Staphylococcal* species
2.	*Lavandula angustifolia* *Pogostemon patchouli* *Citrus bergamia*	Thrush	*Candida albicans*
3.	*Lavandula angustifolia* *Rosmarinus officinalis* *Melaleuca alternifolia*	Thrush	*Candida albicans*
4.	*Lavandula angustifolia* *Anthemis nobilis* *Matricaria chamomiua*	Thrush	*Candida albicans*
5.	*Lavandula angustifolia* *Citrus sinensis* *Cedrus atlantica*	Thrush	*Candida albicans*

Table 7.1 continued Essential oil combinations incorporating *L. angustifolia* in blends with more than two other essential oils.

	Essential oil combination*	Indication for use	Micro-organism responsible for infection
6.	*Lavandula angustifolia* *Cupressus sempervirens* *Melaleuca alternifolia* *Thymus vulgaris*	Trichomonas	*Trichomaniasis vaginalis*
7.	*Lavandula angustifolia* *Hyssopus officinalis* *Cupressus sempervirens*	Non-specific vaginitis	*Gardnerella vaginalis*
8.	*Lavandula angustifolia* *Melaleuca alternifolia* *Melaleuca viridiflora*	Chlamydia	*Chlamydia trachomatis*
9.	*Lavandula angustifolia* *Matricaria chamomiua* *Citrus medica limonum*	General antiseptic	Non-specific
10.	*Lavandula angustifolia* *Melaleuca alternifolia* *Tagetes patula* *Cupressus sempervirens*	Athletes foot	*Trichophyton* species
11.	*Lavandula angustifolia* *Eucalyptus globulus* *Citrus medica limonum* *Melaleuca alternifolia* *Melaleuca viridiflora* *Pinus sylvestrus*	General antiseptic	Non-specific
12.	*Lavandula angustifolia* *Daucus carota* *Thymus vulgaris*	Nail bed infection	*Candida albicans*

* All combinations obtained from the following references: Worwood, 1990; Sellar, 1992; Lawless, 1995; Curtis, 1996; Shealy, 1998; Hili, 2001; Buckle, 2003; Lawrence, 2006.
Shaded= essential oil combinations selected for Design of Experiments analysis.

From Table 7.1 two triple combinations (shaded area) were previously identified in Chapter 2 to be the most promising when combined with *L. angustifolia* at 1:1 ratios and therefore were considered for further investigation. These combinations are *L. angustifolia* in combination with *C. sinensis* and *C. atlantica* for candidal related infections and; *L. angustifolia* in combination with *D. carota* and *M. alternifolia* indicated in the treatment of fungal infections of the nail. The objective of this chapter was therefore to determine if these multiple combinations incorporating *L. angustifolia* displayed antimicrobial activity, and once established to optimise the ratio of the essential oils in the combination for the greatest antimicrobial effect. In order to achieve this, a large number of experiments would need to be conducted, thus the Design of Experiments (DoE) software, MODDE 9.1® was used.

7.1.1. Design of Experiments (DoE) software

Design of Experiments (DoE) software, such as MODDE 9.1®, is used in industry as a means of optimizing experimental outcomes using a limited amount of experiments and resources (Eriksson *et al.*, 2008). When investigating the antimicrobial effects of the essential oils in multiple blends, traditionally a large number of experiments would be required to determine the optimum ratio. DoE structures a systematic experiment outline in order to predict optimum ratios with a limited amount of experiments. A study conducted by Evans *et al*. (2003) aimed to identify if the application of DoE software would be of any value when synthesising chemical compounds. It was identified that the total amount of time spent generating information on the optimum experiment using DoE amounted to three hours, in comparison to two full days in the laboratory to gain the same information.

When investigating the impact of DoE for the optimization of scientific experiments, resources such as Pubmed, ScienceDirect and Scopus search engines were used. In these resources, 488 results were identified for the use of DoE software, with only 25 scientific articles found to be relevant with experiments using MODDE® DoE software (search date: June 2012). In the identified articles, six distinct fields were determined for the use of this software (Table 7.2).

Table 7.2 Design of Experiments software used in industry.

Scientific field	DOE software used	Reference
Pharmacy	MODDE 3.0	Bodea and Leucuta, 1997
	MODDE 3.0	Persson and Åström, 1997
	MODDE 4.0	Bjerregaard *et al.*, 1999
	not specified	Ronkainen *et al.*, 1999
	MODDE 6.0	Brunnkvist *et al.*, 2004
	MODDE 5.0	Viljanen *et al.*, 2005
	MODDE 6.0	Cano *et al.*, 2006
	MODDE 7.0.0.1	Elfstrand *et al.*, 2007
	MODDE 5.0	Bigan *et al.*, 2008
	MODDE 6.0	Verma *et al.*, 2008
	MODDE 8.0	Tosi *et al.*, 2009
	not specified	Snorradóttir *et al*., 2011
	not specified	Colson *et al.*, 2011
Organic chemistry	MODDE 3.0	McKie and Lepeniotis, 1998
	MODDE 3.0	Weckhuysen *et al*., 2000
	MODDE 6.0	Evans *et al*., 2003

Table 7.2 continued Design of Experiments software used in industry.

Scientific field	DOE software used	Reference
Organic chemistry	MODDE 6.0	Gullberg *et al*., 2004
	MODDE 7.0	McNamara *et al*., 2004
	MODDE 8.0	Pedrosa and Bradley, 2008
	MODDE 7.0	Yeber *et al*., 2009
Microbiology	not specified	Roebuck *et al.*, 1995
	not specified	Nerbrink *et al*., 1999
Food preservation	MODDE 4.0	Yann *et al.*, 2005
Dairy farming	MODDE 6.0	Wormbs *et al.*, 2004
Mechanical engineering	not specified	Smith, 2011

The field of pharmacy far outweighed the other disciplines in the number of studies conducted using this software, with 52.0% of the publications identified attributed to this area of study. This outcome is expected, as MODDE® is particularly useful for complex experiments since it provides the researcher an organised approach using a limited amount of resources (Eriksson *et al*., 2008). The use of DoE software has been established since the twentieth century with increasing popularity for its use in pharmaceutical research, only having developed in recent years (Tye, 2004). To the best of my knowledge, no research has been conducted on the optimization of essential oil combinations using MODDE® DoE software.

7.2. Results and Discussion

7.2.1. Model validity for the combinations investigated

Two combinations i.e. *L. angustifolia*: *C. sinensis*: *C. atlantica* and *L. angustifolia*: *D. carota*: *T. vulgaris* were analysed in the worksheets created by MODDE® and the MIC values for each ratio determined against the micro-organism *C. albicans*. Results from laboratory experiments were inserted into the MIC columns (Tables 7.3 and 7.4).

Table 7.3 The MIC values identified for the combination of *L. angustifolia*: *C. sinensis*: *C. atlantica* against *C. albicans*.

Exp No	Run Order	Volume of essential oil (µl)			MIC (mg/ml)
		L. angustifolia	*C. sinensis*	*C. atlantica*	
1	22	0	0	0	0.00
2	23	100.0	0	0	2.85
3	10	0	100.0	0	2.85
4	13	50.0	50.0	0	3.80
5	9	0	0	100.0	3.80
6	24	50.0	0	50.0	2.85
7	12	0	50.0	50.0	2.85
8	18	33.3	33.3	33.3	3.80
9	21	33.3	33.3	33.3	3.80
10	17	33.3	33.3	33.3	3.80
11	16	33.3	33.3	33.3	3.80
12	6	0	0	0	0.00
13	1	100.0	0	0	2.85
14	3	0	100.0	0	2.85
15	5	50.0	50.0	0	3.80
16	2	0	0	100.0	3.80
17	14	50.0	0	50.0	2.85
18	4	0	50.0	50.0	2.85
19	15	33.3	33.3	33.3	3.80
20	7	33.3	33.3	33.3	3.80
21	8	33.3	33.3	33.3	3.80
22	19	33.3	33.3	33.3	3.80

Upon investigation of the results for the combination of *L. angustifolia*: *C. sinensis*: *C. atlantica*, it was noted that the essential oils individually and in combination demonstrated moderate antimicrobial effects. The MIC results obtained for the essential oils are congruent with the results reported in Chapter 4. According to the MIC results obtained, one could assume that no single essential oil has a greater antimicrobial potential over another in the combination. This assumption is made based on the premise that each MIC value is in one well dilution difference suggesting similar antimicrobial effects. This however, was not identified by the MODDE® software, as according to the co-efficient plot for this combination the essential oil *L. angustifolia* was determined as the most significant of the essential oils in the combination, while *C. atlantica* was identified as the least significant (Figure 7.1).

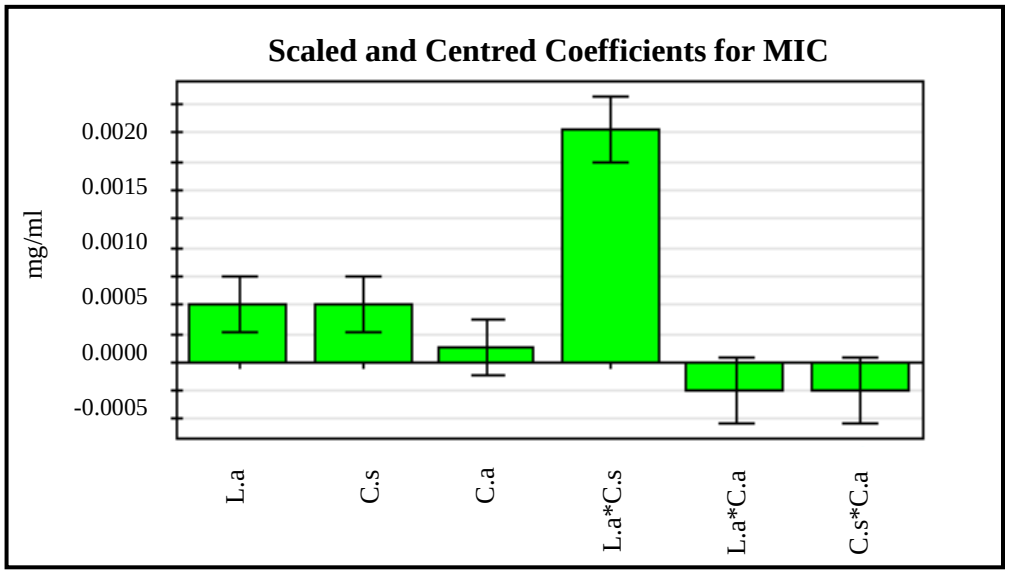


Figure 7.1 The co-efficient plot for the combination of *L. angustifolia* (L.a): *C. sinensis* (C.s): *C. atlantica* (C.a) against *C. albicans*.

Interestingly, the combination of *L. angustifolia* and *C. sinensis* was predicted by MODDE® as having an antagonistic relationship in the triple combination and as such, this interaction was considered responsible for the poor antimicrobial effect determined for the triple combination. The combination of *L. angustifolia* and *C. sinensis* was identified in previous chapters as being the most promising of the combinations investigated with synergy identified at varying concentration ratios of the essential oils against the micro-organisms *C. albicans* (ΣFIC of 0.42) and *S. aureus* (ΣFIC of 0.38).

For the combination of *L. angustifolia*: *D. carota*: *T. vulgaris* a similar antimicrobial effect was observed where only one oil (*T. vulgaris*) was identified as having noteworthy antimicrobial activity (Table 7.4).

Table 7.4 The MIC values identified for the combination of *L. angustifolia*: *D. carota*: *T. vulgaris* against *C. albicans*.

Exp No	Run Order	Volume of essential oil (µl)			MIC (mg/ml)
		L. angustifolia	*D. carota*	*T. vulgaris*	
1	22	0	0	0	0.00
2	23	100.0	0	0	2.85
3	10	0	100.0	0	2.14
4	13	50.0	50.0	0	2.85
5	9	0	0	100.0	1.60
6	24	50.0	0	50.0	2.85
7	12	0	50.0	50.0	2.14

Table 7.4 continued The MIC values identified for the combination of *L. angustifolia*: *D. carota*: *T. vulgaris* against *C. albicans*.

Exp No	Run Order	Volume of essential oil (µl)			MIC (mg/ml)
		L. angustifolia	*D. carota*	*T. vulgaris*	
8	18	33.3	33.3	33.3	2.14
9	21	33.3	33.3	33.3	2.14
10	17	33.3	33.3	33.3	2.14
11	16	33.3	33.3	33.3	2.14
12	6	0	0	0	0.00
13	1	100.0	0	0	2.85
14	3	0	100.0	0	2.14
15	5	50.0	50	0	2.85
16	2	0	0	100.0	1.60
17	14	50.0	0	50.0	2.85
18	4	0	50.0	50.0	2.14
19	15	33.3	33.3	33.3	2.14
20	7	33.3	33.3	33.3	2.14
21	8	33.3	33.3	33.3	2.14
22	19	33.3	33.3	33.3	2.14

These results were found to be congruent with previous results presented in Chapter 4 (See Table 4.4). According to the co-efficient plot generated by the MODDE® software (Figure 7.2) for this combination, *T. vulgaris* oil was considered the most significant of the oils in the combination, while *L. angustifolia* oil was identified as the least significant.

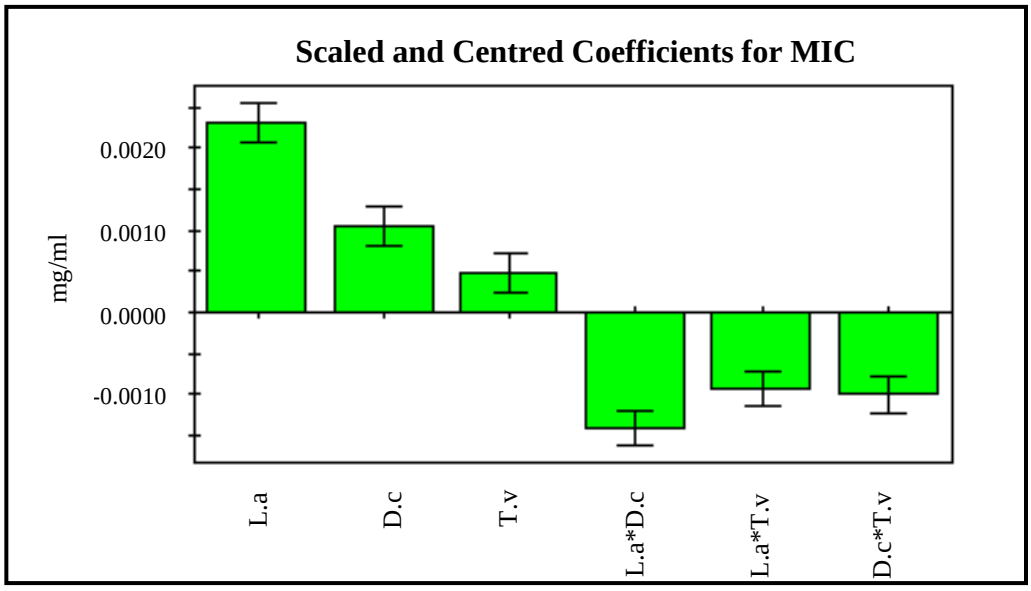


Figure 7.2 The co-efficient plot for the combination of *L. angustifolia* (L.a): *D. carota* (D.c): *T. vulgaris* (T.v) against *C. albicans*.

The combination of *L. angustifolia* and *D. carota* was predicted by MODDE® as having the most significant interaction of the 1:1 essential oil combinations in the triple combination tested. The combination of *L. angustifolia* and *D. carota* was identified in previous chapters (See Chapter 4 Table 4.4) as being significantly synergistic when investigated against *C. albicans* (ΣFIC of 0.50). This result thus further augments the positive interaction of these essential oils identified in previous chapters.

According to further investigations made by the MODDE® software of the MIC results obtained, it was determined that the data in the analysis of the combinations of *L. angustifolia*: *C. sinensis*: *C. atlantica* and *L. angustifolia*: *D. carota*: *T. vulgaris* were to be considered trustworthy. This was determined by the summary of fit plot for the combinations (Figure 7.3). From the summary of fit plot created for the combination of *L. angustifolia*: *C. sinensis: C. atlantica* (Figure 7.3a) it was identified that the combination obtained a R^2 value of 99.3%. This value is significant as the results obtained are considered to be trustworthy if the R^2 value is greater than 30.0%. This states that any possible error in the data obtained for this combination can be explained by the software and as such, future predictions can be made. This combination also demonstrated a high level of predictability as Q^2 obtained a value of 95.5%. The model validity is not applicable as the combination obtained a pure fit with zero pure error, while the model reproducibility was determined to be 100% suggesting that all results obtained can be easily reproduced. A summary of fit plot was also created for the combination of *L. angustifolia*: *D. carota*: *T. vulgaris* (Figure 7.3b) from which it can be identified that the data obtained for the combination demonstrates a good level of confidence as the value for R^2 is 98.5%. This combination also possesses a high level of predictability as Q^2 obtained a value of 90.6%. The summary of fit plot also indicated that the results obtained are highly reproducible.

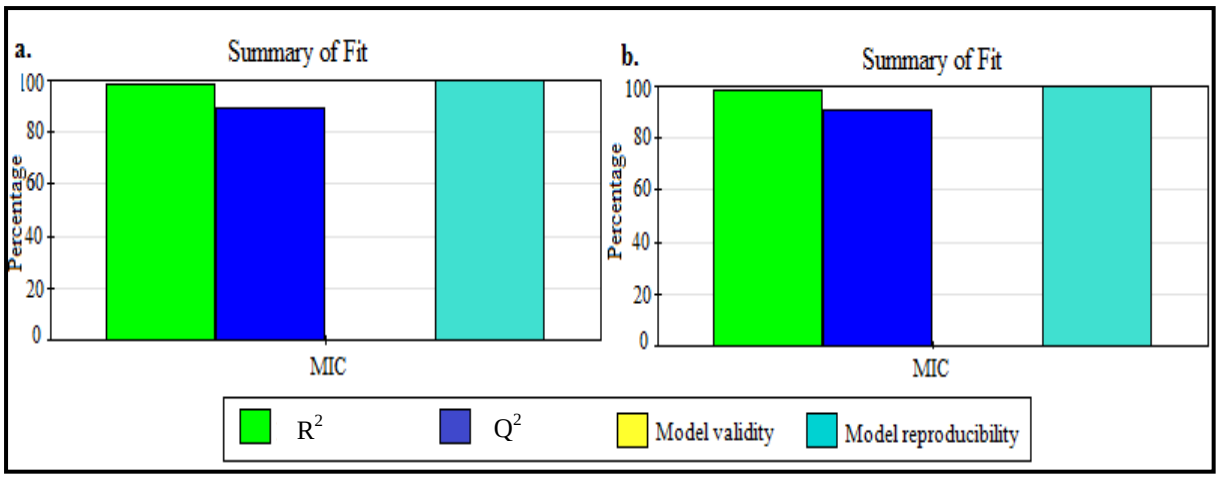


Figure 7.3 The summary of fit plot for the combination of *L. angustifolia*: *C. sinensis: C. atlantica* (a) and the summary of fit plot for the combination of *L. angustifolia*: *D. carota*: *T. vulgaris* (b).

These findings were further augmented by the observed versus predicted plot for these combinations (Figure 7.4). According to the results obtained for the combination of *L. angustifolia*: *C. sinensis: C. atlantica* in the observed vs. predicted plot (Figure 7.4a) it can be identified that this model has a strong ability to predict future outcomes for this combination, as the majority of the combinations are found tightly plotted around the regression line as well as on top of one another. The same was identified for the combination of *L. angustifolia*: *D. carota*: *T. vulgaris* (Figure 7.4b). This outcome was to be expected due to the high Q^2 values previously identified.

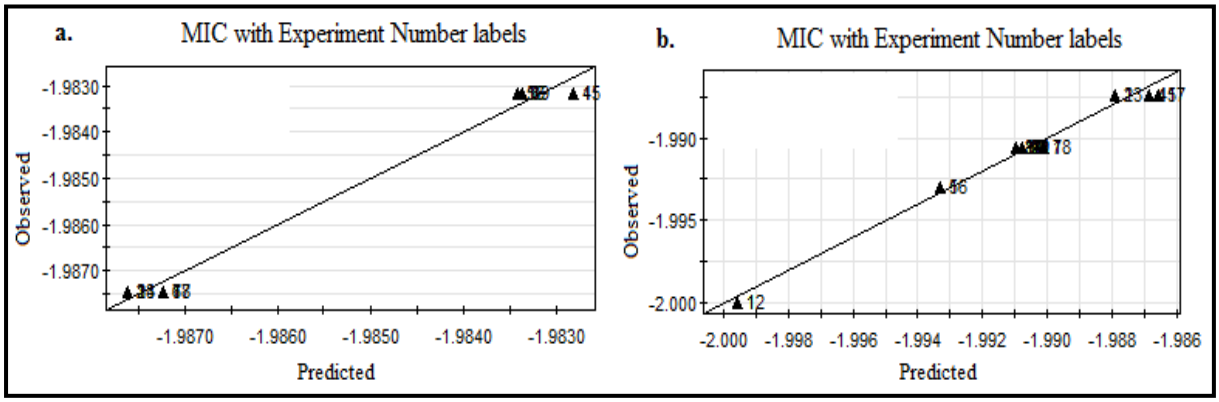


Figure 7.4 The observed versus predicted plot for the combination of *L. angustifolia*: *C. sinensis: C. atlantica* (a) and the observed versus predicted plot for the combination of *L. angustifolia*: *D. carota*: *T. vulgaris* (b).

Selections of graphs were chosen for discussion due to their specificity in interpreting the interaction of the essential oils in the combination, however, for the sake of brevity the remaining data is summarised in Table 7.5.

Table 7.5 Summary of results obtained for the combinations tested.

Graph	**Requirements**	***L. angustifolia*: *C. sinensis*: *C. atlantica***	***L. angustifolia*: *D. carota*: *T. vulgaris***
Replicate plot	Distribution of replicates	No variation between replicates of the same experiment	No variation between replicates of the same experiment
Histogram	Transformation required	Yes	Yes
Residual N-plot	Distribution of points	Along regression line	Along regression line

This data created for the combinations tested was formed from a limited number of experiments (22 runs per combination), yet this data gives a large amount of comprehensive information on the interactions between the essential oils investigated. This outcome further augments the popularity of this software in research as the amount of information obtained greatly exceeds the amount of time and resources used to obtain it.

Based on the information given for the combinations of *L. angustifolia*: *C. sinensis:* *C. atlantica* and *L. angustifolia*: *D. carota*: *T. vulgaris* (Figures 7.1 to 7.4 and Tables 7.3 to 7.5), further analysis by means of response contour plot formation can be created and tested, as the Design of Experiments software has been determined as having a high level of accuracy for future predictions.

7.2.2. Response contour plot

Once the validity of the MIC results obtained for the combinations were confirmed, the MODDE® software was used to generate a response contour plot per combination (Figures 7.5 and 7.6). The response contour plots indicate varying concentrations of *L. angustifolia* is added to the triple combinations. Initially no *L. angustifolia* essential oil is added to the combination (Figures 7.5a and 7.6a), then in the second graph *L. angustifolia* is added to the combination at a volume of 50 µl (Figures 7.5b and 7.6b). Finally, *L. angustifolia* is added to the triple combinations at a volume of 100 µl (Figures 7.5c and 7.6c).

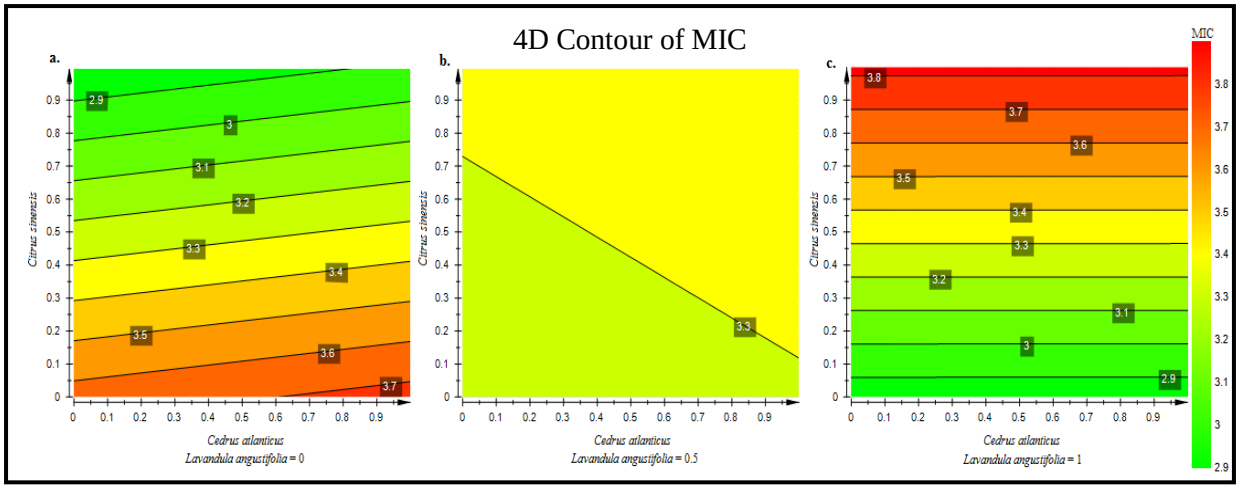


Figure 7.5 The response contour plot for the combination of *L. angustifolia*: *C. sinensis: C. atlantica* for varying concentrations of *L. angustifolia*.

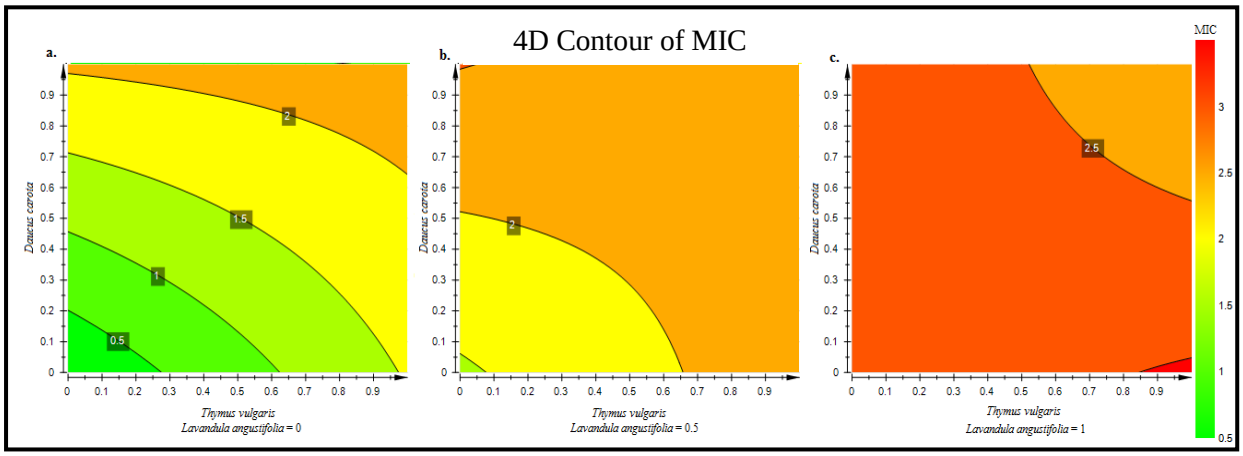


Figure 7.6 The response contour plot for the combination of *L. angustifolia*: *D. carota*: *T. vulgaris* for varying concentrations of *L. angustifolia*.

7.2.2.1. Response contour plot for the combination of *L. angustifolia*: *C. sinensis: C. atlantica*

According to the response contour plot of the combination of *L. angustifolia*: *C. sinensis: C. atlantica* (Figure 7.5) it is apparent that where no *L. angustifolia* essential oil is added to the combination, a greater antimicrobial effect is maintained where *C. sinensis* is in greater concentration to *C. atlantica*. This outcome is incredibly interesting, as according to the MIC values obtained for these essential oils in Chapter 4, this result would be predicted, as *C. sinensis* demonstrates a far greater antimicrobial effect against the micro-organism

C. albicans (MIC value of 2.00 mg/ml) than *C. atlantica* (MIC value of 4.00 mg/ml). When 100 µl of *L. angustifolia* is added to the combination, it is noted that the lower the concentration of *C. sinensis* essential oil and the greater the concentration of *C. atlantica* essential oil in the combination, the greater the antimicrobial potential of the combination. This outcome further augments the findings of the co-efficient plot for this combination (Figure 7.1) as the interaction between *L. angustifolia* and *C. sinensis* essential oil is predicted to reduce the antimicrobial potential of the combination.

From the response contour plot of this combination, five random points were selected for confirmatory analysis by means of MIC determination using the micro-titre plate assay (Table 7.6). This was done to confirm the predictability of the software in order to determine the trustworthiness of the optimal essential oil ratio to be identified later.

Table 7.6 MIC results (n=2) observed after confirmatory analysis of predicted results for the combination of *L. angustifolia*: *C. sinensis: C. atlantica*.

Experiment run	Ratios			Predicted MIC (mg/ml)	Observed MIC (mg/ml)
	L. angustifolia	***C. sinensis***	***C. atlantica***		
1a	28	27	45	2.62	2.85
2a	70	15	15	2.61	2.14
3a	24	30	46	3.43	2.85
4a	31	13	56	2.60	2.14
5a	33	33	33	3.30	3.80

From the table (Table 7.6) it is apparent that the Design of Experiments (MODDE 9.1®) software possesses a high level of predictability for the given combination as the predicted results are confirmed in one well dilution.

These predicted MIC values for the combination further indicate a lack of noteworthy antimicrobial effect. The combination of the essential oils in equal parts is predicted to give an MIC value of 3.30 mg/ml. This value is higher than that obtained for the essential oils of *C. sinensis* and *C. atlantica* in combination with *L. angustifolia* in a 1:1 ratio, as MIC values of 1.00 mg/ml were obtained for both combinations (Chapter 4). This outcome suggests that the use of this particular triple combination is not as beneficial in the treatment of fungal infections as the combination of *L. angustifolia* and *C. sinensis* and *L. angustifolia* in combination with *C. atlantica* in 1:1 blends.

7.2.2.2. Response contour plot for the combination of *L. angustifolia*: *D. carota*: *T. vulgaris*

For the combination of *L. angustifolia*: *D. carota*: *T. vulgaris* it is apparent that the greater the concentration of *L. angustifolia* in the combination, the less the antimicrobial effect (Figure 7.6). According to the response contour plot for this combination, where *L. angustifolia* is absent in the blend, it should be noted that the greater the concentration of *T. vulgaris* in the combination the greater the antimicrobial effect. This outcome suggests that *T. vulgaris* plays a greater role in the antimicrobial interaction of the essential oil combination of *D. carota* and *T. vulgaris.* This outcome is very interesting, as it augments previous findings on the antimicrobial activity of these essential oils (Chapter 4) as well as further strengthens the results obtained in the co-efficient plot for this combination (Figure 7.2). When *L. angustifolia* is added to the combination, the antimicrobial potential of the combination diminishes.

From the response contour plot of this combination, five random points were selected for confirmatory analysis by means of MIC determination using the micro-titre plate assay (Table 7.7).

Table 7.7 MIC results (n=2) observed after confirmatory analysis of predicted results for the combination of *L. angustifolia*: *D. carota*: *T. vulgaris*.

Experiment run	Ratios			Predicted MIC (mg/ml)	Observed MIC (mg/ml)
	L. angustifolia	*D. carota*	*T. vulgaris*		
1b	37	29	34	2.04	2.14
2b	35	25	40	2.06	1.60
3b	31	32	37	2.13	1.60
4b	24	35	41	2.20	2.14
5b	33	33	33	2.10	2.14

According to Table 7.7, the results observed confirm those that were predicted by the Design of Experiments (MODDE 9.1®) software.

This triple combination of *L. angustifolia*: *D. carota*: *T. vulgaris* is not as effective as the 1:1 combinations of *L. angustifolia* with the essential oils of this combination

7.3. Overview

Essential oils are often applied in combinations of up to seven individual oils for the treatment of microbial infections (Alternative Therapies, 2005). The most popular essential oil combination available on the market is Thieves® essential oil blend. This blend is comprised of 17 parts cinnamon bark, 29 parts lemon, 33 parts clove bud, 8 parts rosemary and 13 parts eucalyptus oil. The assumed antimicrobial uses of this essential oil blend ranges from the treatment of general cuts and wounds to the alleviation of lung consolidation and respiratory infections (Carey, 2007).

Two triple combinations were chosen for analysis by means of Design of Experiments software. The combination of *L. angustifolia*: *C. sinensis*: *C. atlantica* was indicated in the treatment of thrush, while *L. angustifolia*: *D. carota*: *T. vulgaris* was specified for use in combination in the treatment of yeast infections of the nail bed. The MIC screening of these essential oils in 1:1:1 combinations demonstrated a lack of noteworthy antimicrobial effect. Therefore the combinations required further investigation in order to optimise the potential antimicrobial efficacy of these triple combinations. In order to do this a large number of experiments would need to be conducted incorporating the use of limited resources. This was overcome by means of Design of Experiments (DoE) software.

Many studies have been conducted in the pharmaceutical industry for the optimisation of drug formulations by means of DoE software. A study conducted by Bodea and Leucuta (1997) aimed to optimise a hydrophilic matrix tablet for the release of an active ingredient by investigating the varied effects of two excipients using DoE software. By means of MODDE® 3.0 software, an optimised drug formulation was predicted for the controlled release of active ingredient from the hydrophilic tablet. The observed response was as predicted by the software and thus the formulation optimized. The future use of this software was greatly encouraged by the researchers of this study. Another study conducted by Cano *et al*. (2006) aimed to optimise the xylan degradation activity of monolithic enzymatic membranes by means of their composition using MODDE® 6.0 DoE software. By means of the DoE software, predictions to the best membrane composition for this outcome were given and their accuracy determined by additional experiments. According to Cano *et al*. (2006), DoE proved to have a high level of predictive efficacy by successfully optimising the

membrane composition for xylan degradation. The researchers have also greatly encouraged the use of DoE software for future analysis.

According to the research conducted in this study, MODDE® DoE software has proven to have a high level of predictability with all MIC values observed for the combinations predicted to be in one well dilution of the predicted value (Figure 7.7).

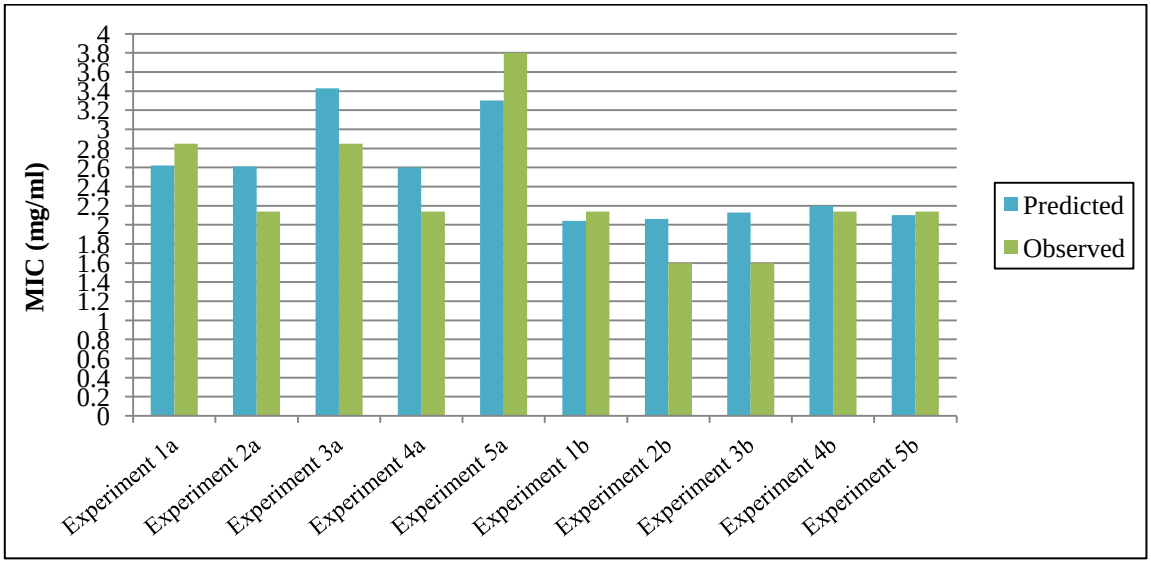


Figure 7.7 The MIC predictability efficacy of MODDE 9.1® software for triple combinations investigated. Experiments 1a- 5a are found in more detail in Table 7.6 and Experiments 1b-5b are found in more detail in Table 7.7.

For the combinations investigated it was determined that no one particular essential oil in the combination was responsible for a more efficient antimicrobial effect in the combination. Effectively, all the essential oils in the combination were determined as having equalling antimicrobial effects as predicted values determined by the MODDE® software did not vary considerably even when essential oil volumes did. A possible explanation for this outcome is that the essential oils chosen for analysis all demonstrated similar antimicrobial activity. This similarity in antimicrobial activity resulted in the available window for prediction to be greatly limited. The software allows the user to set a value to which a prediction should be made. Although, in this analysis, a prediction value of 0.50 mg/ml was selected, the software could not adequately predict to this value due to the similarities in MIC values of the individual and combined essential oils. This outcome is determined as a limitation to the use of this software in the optimization of essential oils for antimicrobial purposes.

Therefore, DoE software could hold great promise in the prediction of antimicrobial combinations where larger numbers of combinations are used, as well as where antimicrobial activity is unknown. This software is also of immense value where the amount of sample available for investigation is limited and therefore the analysis undertaken is required to be specific and efficient. Therefore, although DoE software indicates limitations to its use when optimising antimicrobial effects, the promise of this tool for prediction use has potential and thus should be considered for use in future triple combination analysis. However, the values determined for the optimal combinations should only be used as a guide and rather interpreted as determining the more efficient sample in the combinations for the required response.

7.4. General conclusion

- The Design of Experiments (MODDE 9.1®) software identified that *L. angustifolia* (from the combination of *L. angustifolia*: *C. sinensis*: *C. atlantica*) and *T. vulgaris* (from the combination of *L. angustifolia*: *D. carota*: *T. vulgaris*) were the essential oils with the greater antimicrobial effect in the combinations analysed.

- The Design of Experiments (MODDE 9.1®) software also identified that *C. atlantica* (from the combination of *L. angustifolia*: *C. sinensis*: *C. atlantica*) and *L. angustifolia* (from the combination of *L. angustifolia*: *D. carota*: *T. vulgaris*) were the essential oils with the least antimicrobial effect in the combinations analysed.

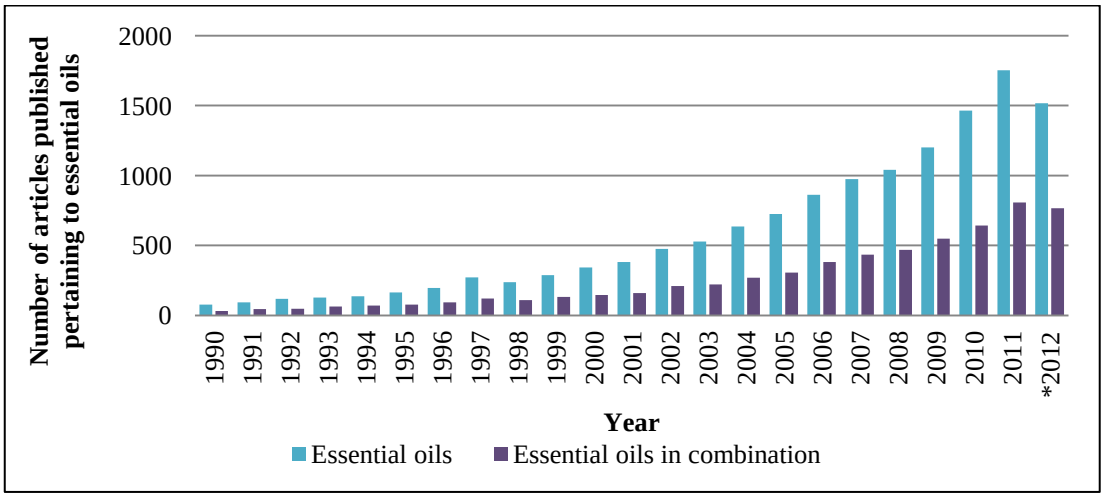
- According to the response contour plot for the combination of *L. angustifolia*: *C. sinensis*: *C. atlantica*, combinations higher in *L. angustifolia* oil are predicted to produce the best MIC response. While for the combination of *L. angustifolia*: *D. carota*: *T. vulgaris*, when *L. angustifolia* is added to the combination, the antimicrobial potential of the combination diminishes.

- The predictability of MODDE 9.1® software has been proven to be effective and as such holds promise for future use.

CHAPTER 8
GENERAL DISCUSSION, CONCLUSION AND FUTURE RECOMMENDATIONS

8.1. General discussion

The antimicrobial properties of essential oils have been exponentially studied in the last 15 years (Figure 8.1). When analysing of the literature (search engines Pubmed, ScienceDirect and Scopus) pertaining to the antimicrobial effects of essential oils it was identified that the field of essential oil analysis has grown in popularity over recent years with 273 published articles available for review (search date: July, 2012). However, only 6.5% of the research conducted on essential oils is attributed to their antimicrobial effects when placed in combination. The results presented in this thesis have demonstrated the antimicrobial effects of 54 individual essential oils as well as their antimicrobial effects when studied in combination with *L. angustifolia*. *L. angustifolia* was identified as the most popularly combined essential oil in aromatherapy. Essential oils of triple combinations were also investigated using Design of Experiments software, the first of this kind of analysis of essential oil combinations. Furthermore, the major chemical constituents of the essential oils investigated were reported and found to be in agreement with literature previously reported.



* Number of publications for 2012 until July.

Figure 8.1 Number of scientific publications available for review on Pubmed, ScienceDirect and Scopus pertaining to the antimicrobial properties of essential oils.

8.2. Thesis summary

L. angustifolia essential oil in combination with 54 other essential oils was investigated in order to establish some antimicrobial evidence for the use of essential oils in combination in the field of aromatherapy. Figure 8.2 summarises the findings of this research with a more extensive discussion followed based on the objectives of this study outlined in Chapter 1.

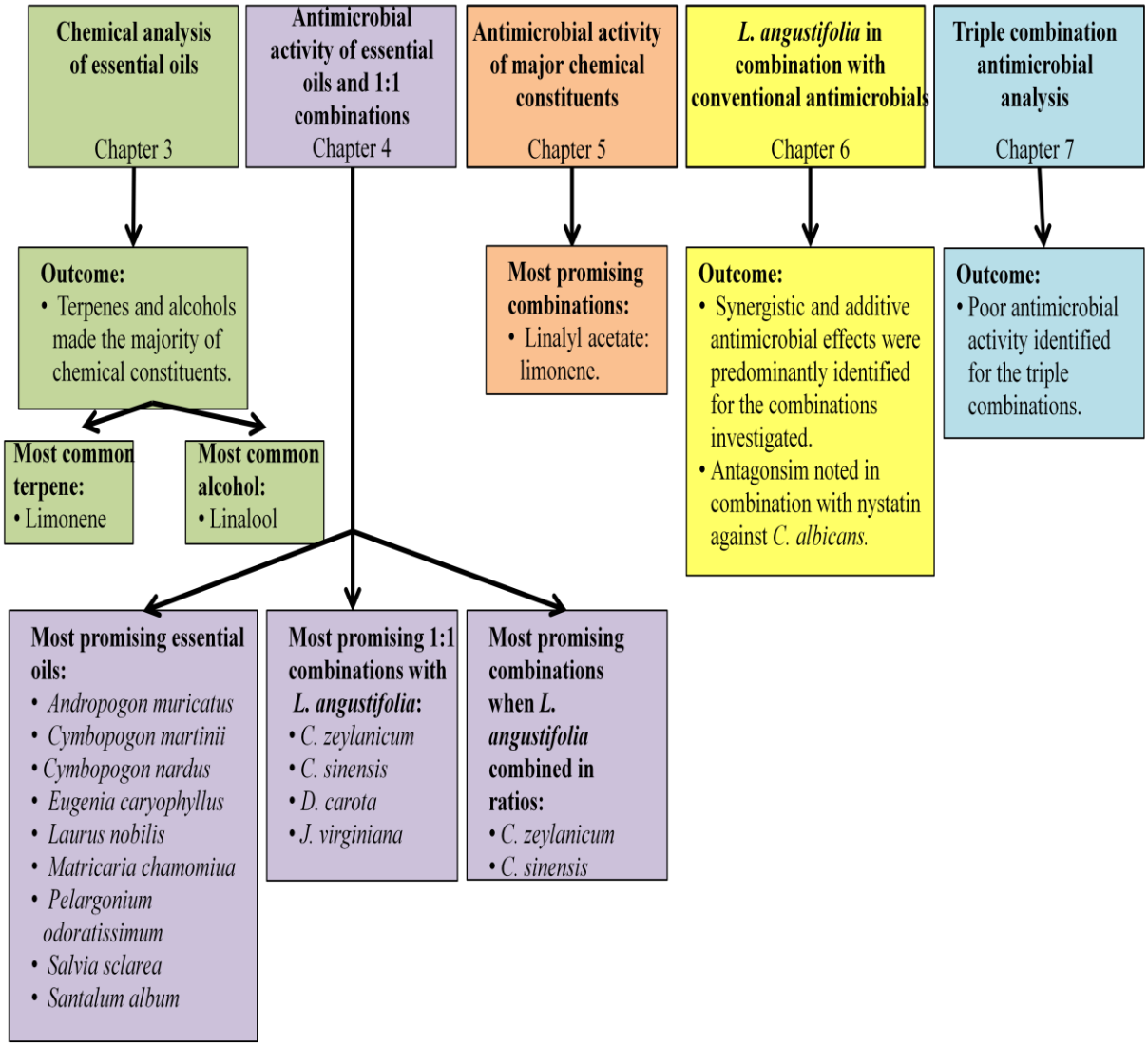


Figure 8.2 Summary of thesis and results obtained.

Objective 1: To determine the composition of *Lavandula angustifolia* and the essential oils of its identified combinations using gas chromatography coupled with mass spectrometry (GC-MS) apparatus.

The chemical composition of all 54 essential oils investigated was determined by means of GC-MS and the major chemical constituents presented in Chapter 3 (Table 3.1). The class of terpenes and alcohols demonstrated the greatest level of occurrence with 87.7% of the essential oils investigated comprising of a chemical constituent in this class. The most frequently identified chemical constituents in this class were limonene (terpene) and linalool (alcohol). This outcome is expected as the class of chemical compounds, terpenes, is one of the most abundant in essential oils (Clarke, 2008).

Objective 2: To determine the antimicrobial activity of *Lavandula angustifolia* and commonly combined aroma-therapeutic essential oils using minimum inhibitory concentration (MIC) antimicrobial assays.

When investigating the antimicrobial effects of *L. angustifolia* against 14 pathogens, it was identified that *L. angustifolia* demonstrated a 90% noteworthy (MIC values of 2.00 mg/ml or lower) antimicrobial effect. The remainder of the oils in the data set demonstrated 76% noteworthy antimicrobial effects against *C. albicans*, *S. aureus* and *P. aeruginosa.* The essential oils demonstrating the highest antimicrobial activities in this study are given in Table 8.1. The antimicrobial activities of these essential oils are congruent with previous findings in literature.

Table 8.1 The essential oils demonstrating the highest antimicrobial activities in this study.

Essential oil	Micro-organism	MIC (mg/ml)	Previous research conducted
Andropogon muricatus	*S. aureus* (ATCC 6538)	0.75	No previous research conducted on this essential oil against *S. aureus*
Cymbopogon martinii	*C. albicans* (ATCC 10231)	0.75	Agarwal *et al*., 2008
Cymbopogon nardus	*C. albicans* (ATCC 10231)	0.75	Hammer *et al*., 1999
Eugenia caryophyllus	*C. albicans* (ATCC 10231)	0.50	Agarwal *et al*., 2008; Agarwal *et al*., 2010

Table 8.1 continued The essential oils demonstrating the highest antimicrobial activities in this study.

Essential oil	Micro-organism	MIC (mg/ml)	Previous research conducted
Laurus nobilis	*C. albicans* (ATCC 10231)	0.75	Dadalioğlu and Evrendilek, 2004; Al-Hussanni and Mahasneh, 2011
	S. aureus (ATCC 6538)	0.83	
Matricaria chamomiua	*C. albicans* (ATCC 10231)	0.50	Agarwal *et al*., 2008; Agarwal *et al*., 2010
Pelargonium odoratissimum	*C. albicans* (ATCC 10231)	0.75	No previous research conducted on this essential oil against *C. albicans*
Salvia sclarea	*C. albicans* (ATCC 10231)	0.88	Hammer *et al*., 1999; Peana *et al*., 1999
Santalum album	*S. aureus* (ATCC 6538)	0.25	Hammer *et al*., 1998; Hammer *et al*., 1999; Giriram *et al*., 2006
	P. aeruginosa (ATCC 27858)	0.50	

Previous to this study, no research has been conducted on the antimicrobial efficacy of *P. odoratissimum* against the micro-organism *C. albicans*; and *A. muricatus* against the micro-organism *S. aureus.* The results observed from the microbial activity of the essential oils have demonstrated the potential of these oils for antimicrobial purposes.

Objective 3: To determine the antimicrobial interaction of *L. angustifolia* essential oil with the other 54 essential oils at 1:1 concentrations (resulting in the calculation of the fractional inhibitory concentration (ΣFIC)) and at various concentration combinations (resulting in the formation of isobolgrams).

When placed in equal ratios, it was identified that 23.5% of the combinations were synergistic, 52.5% additive, 23.5% non-interactive and 0.5% antagonistic. The essential oils demonstrating the highest level of synergy against the micro-organisms tested in this study, using MIC methodology, are given in Table 8.2.

Table 8.2 The essential oils demonstrating the highest level of synergy against the micro-organisms tested.

Essential oil combination	ΣFIC		
	C. albicans **(ATCC 10231)**	***S. aureus*** **(ATCC 6538)**	***P. aeruginosa*** **(ATCC 27858)**
L. angustifolia: *C. zeylanicum*	0.40	0.50	0.53
L. angustifolia: *C. sinensis*	0.42	0.38	0.51
L. angustifolia: *D. carota*	0.50	0.50	0.55
L. angustifolia: *J. virginiana*	0.50	0.50	0.56

When placed in varying ratios (*L. angustifolia* in combination with *C. zeylanicum*), mostly synergy was observed. *L. angustifolia* in combination with *C. sinensis* had the greatest antibacterial effect. Previous to this study, no research has been conducted on the combination of these essential oils.

Objective 4: To determine the antimicrobial activity of the major chemical constituents of *L. angustifolia* and essential oils where combinations demonstrate synergistic effects

When the major chemical constituents of the essential oil combinations that demonstrated pronounced synergy were investigated using MIC assay, only one possible interaction was identified where the major compounds had a direct impact on the overall antimicrobial activity. The combination of linalyl acetate (originating from *L. angustifolia*) and limonene (originating from *C. sinensis*) against the micro-organism *C. albicans* demonstrated identical effects to that of the pure essential oils. This relationship suggests that these major chemical constituents are responsible for the antimicrobial effects of the essential oils due to their interaction. This outcome, however, would require further analysis for confirmation.

Objective 5: To determine the antimicrobial activity of *L. angustifolia* essential oil in combination with common conventional antimicrobial agents

L. angustifolia essential oil was placed in combination with four antimicrobial agents (chloramphenicol, ciprofloxacin, fusidic acid and nystatin) and the antimicrobial interaction determined. According to the results obtained it was identified that *L. angustifolia* provided

the pivotal antimicrobial role in the combinations against the micro-organisms *C. albicans* and *S. aureus*, with ratios higher in *L. angustifolia* essential oil concentration showing considerably better antimicrobial effects. When tested against *P. aeruginosa*, the converse was noted with better antimicrobial effects noted for ratios greater in concentrations of the antimicrobial agent. The combination of *L. angustifolia* and chloramphenicol demonstrated the best antifungal effect, while *L. angustifolia* in combination with ciprofloxacin was noted as the greater antibacterial combination.

Objective 6: To optimise triple antimicrobial combinations including *L. angustifolia,* by using the Design of Experiments Software, MODDE 9.1®.

Essential oils used in aromatherapy are commonly placed in blends of unlimited number for a presumed antimicrobial effect, thus Chapter 7 aimed to identify if any possible synergistic antimicrobial effect could be identified for combinations of three essential oils. The study comprised of two triple combinations. The essential oils selected for analysis included *L. angustifolia* in combination with *C. sinensis* and *C. atlantica;* and *L. angustifolia* in combination with *D. carota* and *T. vulgaris*. These combinations were analysed for the first time by means of a novel system approach. The approach implemented was the use of Design of Experiments (MODDE 9.1®) software to optimise the combinations for the best possible antimicrobial effect. According to the software and follow-up MIC assays used to validate the predicted MIC values, both essential oil combinations demonstrated a lack in noteworthy antimicrobial activity. This outcome was incredibly interesting, as each essential oil demonstrated noteworthy antimicrobial effects when used in 1:1 combinations with *L. angustifolia*. The antimicrobial activity of the essential oils used in the triple combinations is summarised in Table 8.3.

Table 8.3 The antimicrobial activity of the essential oils used in the triple combinations.

Essential oil	MIC (mg/ml)		
	Essential oil	1:1 combination*	Triple combination**
C. sinensis	2.00	1.00	3.80
C. atlantica	4.00	1.00	
D. carota	3.00	1.50	2.14
T. vulgaris	1.00	1.00	

* 1:1 combination denotes 50 µl of the essential oil in combination with 50 µl of *L. angustifolia*. ** Triple combination denotes 33.33 µl of each essential oil in the combination with 33.3 µl of *L. angustifolia*.

This result suggests that the antimicrobial effects of essential oils are greater in simpler blends (i.e. 1:1 combinations) however; only two triple combinations were investigated. As such, further analysis on other triple combinations for possible antimicrobial effects needs to be investigated for other combinations to validate this observation.

Typically essential oils used in aromatherapy are combined to bring about a holistic approach to healing (Lawless, 1995). Essential oils are also commonly combined according to certain properties rather than combined specifically for antimicrobial effects. One such property is the concept of "quenching". Quenching is the application of a particular essential oil in a combination for the purpose of suppressing the harmful effects of another essential oil. According to Burfield and Sheppard-Hanger (2005), the concept of quenching is considered to be highly contested with some groups, such as the IFRA (International Fragrance Association) Hazards Working Group, insisting that the phenomena still be considered. Examples of essential oils believed to perform the action of quenching when in combination are *Cymbopogon citrates* and *Citrus paradisi*. *C. citrates* is comprised of the chemical constituent, citral, responsible for dermal irritation when the essential oil is applied topically. When *C. paradisi* is added to a combination containing *C. citrates*, the major chemical constituent limonene quenches this effect, rendering the combination safe for use (Clarke, 2008). The quenching effects of essential oils are further augmented by studies conducted by Opdyke, 1975; Opdyke, 1976; Opdyke, 1978; Opdyke, 1982; Allenby *et al*., 1984; Guin *et al*., 1984; Karlberg *et al*., 2001 and Nilsson *et al*., 2004.

Another reason for the combination of certain essential oils is for the unique fragrance created by the combination. Combinations such as frankincense and ginger are considered to be over-powering, as each oil has a very strong odour and as such are not considered for use

in combination. The combination of lavender and rosemary is considered more pleasant with the mixture of these oils delivering a synergistic aroma (McGilvery and Reed, 1995). The sense of smell is thought to provoke memory and emotion and as such is responsible for a holistic sense of healing (Clarke, 2008).

Essential oils are also placed in combination for a variety of other pharmacological reasons. The combination of lavender, sweet marjoram, patchouli and vetiver in an aqueous cream base were tested for effectiveness in treating dementia associated symptoms. A double blind clinical trial of 56 geriatric patients was performed whereby the essential oils were applied via massage to limbs five times a day for four weeks. The placebo applied was the use of aqueous cream void of essential oil. The group massaged with essential oil based aqueous cream demonstrated a significant relief in dementia symptoms (Bowles-Dilys *et al*., 2002). Essential oil combinations have also been suggested for use in the treatment of diabetes (Talpur *et al*., 2005), anxiety and stress (Edris, 2007), insomnia (Diego *et al*., 1998), functional dyspepsia (Bașer and Buchbauer, 2010), nausea (Gilligan, 2005) and pain (Han *et al*., 2006).

These examples provide some alternate reasons for combining oils and when examining antimicrobial validations, should not be ignored as the use of blends for infectious diseases may extend to various other additional applications.

8.3. Future recommendations

This study has highlighted the antimicrobial effectiveness of essential oils in combination, and as such has identified a largely uninvestigated area for future studies. A number of recommended strategies have thus been included for future consideration.

8.3.1. Toxicity studies

The premise that a product originating from nature is safe to use is not founded on truth as many natural products have toxic effects (Bateman *et al*., 1998; Stickel *et al*., 2000).

L. angustifolia, as stated in previous chapters, is believed to be the most popular of all essential oils used in aromatherapy as it has no known toxicity. According to Prashar *et al.* (2003) this claim is inaccurate as more and more cases of skin sensitivity and allergic reactions have been reported. Prashar *et al.* (2003) aimed to identify the cytotoxicity of *L. angustifolia* essential oil. Solutions of *L. angustifolia* essential oil were tested against three main skin cell types, 153BR (fibroblasts), HNDF (human normal dermal fibroblasts) and HMEC-1 (human dermal microvascular endothelial cell) using the neutral red (NR) assay. It was identified that the cytotoxic effects of *L. angustifolia* oil increased with an increase in concentration, as cell viability changed from 80-100% at a concentration 0.125% to 50% (v/v).Thus the premise that *L. angustifolia* essential oil is "safe" and can be used directly on the skin undiluted is hugely incorrect. Essential oils, as natural products, consist of a variety of chemical compounds. Many of these compounds are not deemed safe for topical or internal use in humans due to their high level of toxicity. Table 8.4 is taken from data compiled by Price and Price (1992) in which the toxicity of an essential oil is determined for an average 70 kg test subject. These volumes are quite significant; suggesting that overdose of essential oils may not be an easy feat to achieve when using them for therapeutic reasons (Price and Price, 1992). Certain essential oils have also been identified as having the ability to cause sensitivity to the skin when applied topically. These essential oils typically include cinnamon, oregano, clove and thyme (skin irritation); while thyme and cinnamons have been found to be responsible for mucous membrane irritation due to their high phenol content. Other possible side effects of essential oils include phototoxicity (caused by essential oils of angelica, cedarwood and cumin), neurotoxicity (sage), hepatotoxicity (cedarwood, thyme and tarragon) and nephrotoxicity (birch and sandalwood) (Price and Price, 1992).

Table 8.4 Toxic doses of certain essential oils according to Price and Price (1992).

Essential Oil	Lethal dose (ml)	Essential Oil	Lethal dose (ml)	Essential Oil	Lethal dose (ml)
Lavender	389	Myrrh	128	Hyssop	109
Basil	109	Patchouli	389	Juniper berries	622
Black pepper	389	Pettitgrain	389	Marjoram	174
Cedarwood	389	Peppermint	350	Tea Tree	148
Cinnamon	264	Pine	535	Vetiver	389
Cypress	389	Rosemary	389	Ylang-Ylang	389
Eucalyptus	345	Sage	196	Geranium	389
Fennel	296	Sandalwood	434	Thyme	366

Thus it is recommended that future studies include the evaluation of the toxicology of essential oils with potential antimicrobial effects.

8.3.2. Mechanism of action studies

A compound can have an effect on the integrity of a micro-organism by means of a multitude of pathways, such as by damage to the cellular membrane (Cox *et al*., 2001; Lambert *et al*., 2001; Bouhdid *et al*., 2009), potassium ion leakage (Lambert *et al*., 2001, Prashar *et al*., 2003, Inoue *et al*., 2004), changes in cell morphology (Burt and Reinders, 2003; Kwon *et al*., 2003; Bennis *et al*., 2004), changes in membrane properties (Ultee *et al*., 2002; Ahmad *et al*., 2011), disruption of intracellular pH homeostasis (Breeuwer *et al*., 1996; Turgis *et al*., 2009), disruption of intracellular Ca^{2+} homeostasis (Rao *et al*., 2010), enzyme inhibition (Bang *et al*., 2000; Thoroski, 1989; Luciano and Holley, 2009), inhibition of cell division (Domadia *et al*., 2007; Hemaiswarya *et al*., 2011), changes in toxin production (Ultee and Smid, 2001; de Souza *et al*., 2010) or inhibition of cellular respiration (Inouye *et al*., 1997; Cox *et al*., 2001). Each mechanism is unique with essential oils having the potential to affect any system. Due to the complexity of essential oil chemistry it has been determined that essential oils have the ability to affect any number of pathways. According to Oussalah *et al*. (2006) and Oussalah *et al*. (2007), the essential oil of Spanish oregano (*Coridothymus capitatus)* performs its antimicrobial action through four distinct mechanisms. *Coridothymus capitatus* performs its antimicrobial effect by increasing extracellular ATP, releasing cellular content, reducing intracellular pH and affecting membrane integrity. Although some individual chemical compounds have been determined as having antimicrobial potential independently, the effects of the essential oils are predominantly due to the interactions of the chemical entities comprising them (Hyldgaard *et al*., 2012). When combining essential oils, the same outcome is identified in that certain mechanisms work synergistically to produce a greater antimicrobial effect. Having the knowledge surrounding the effects of essential oils may in future allow for a more systematic and accurate means of approach when combining essential oils for antimicrobial effects, as varying mechanisms of action can be employed for a more synergistic effect.

8.3.3. Stereochemistry

Isomerism, according to Clarke (2008), is "the existence of a compound in the form of molecules with the same molecular formula but a different structural arrangement of the atoms." Stereochemistry is the study of isomerism. Isomerism is a common occurance among essential oils with variances in chemical arrangement responsible for differences in physical and biological properties of isomers. In the body, changes are detected by means of chemical interactions with cell receptors. Due to the differences in chemical arrangement of atoms among isomers, the interaction of the molecules with the receptor sites vary, causing differing biological effects depending on isomers (Clarke, 2008). An example of this phenomenon is noted in the study conducted by van Vuuren and Viljoen (2007) in which the two isomers of limonene were investigated for their antimicrobial potential. From the study it was determined that (-)-limonene was the more active isomer with inhibition shown against five of the eight pathogens evaluated, while the (+)-limonene isomer showed only moderated antimicrobial potential. This was further augmented as (-)-limonene showed a three times greater antimicrobial effect against *S. aureus* when compared to (+)-limonene. Therefore, it is apparent that antimicrobial activity of essential oils is greatly affected by the stereochemistry of the chemical compounds and as such, should be considered when evaluating the antimicrobial potential of essential oils and their major chemical constituents. Some of the molecules assayed in Chapter 5 are chiral compounds (e.g. linalool and limonene). Further studies could investigate the effect of chirality on antimicrobial activity by combining single enantiomers (+ or -) or the racemic mixtures in various ratios.

8.3.4. Essential oil combinations of greater than two

According to the findings of this research, poor antimicrobial effects were determined for essential oils combinations of greater than two. This however, was based on the finding of only two triple essential oil combinations. As previously indicated, the antimicrobial activity of essential oils in combination has been poorly investigated and as such multiple combination analysis is an unknown field. With the use of design systems such as MODDE 9.1®, the amount of time and resources spent on analysis is greatly reduced with an unlimited amount of variables for inclusion in analysis (Eriksson *et al.*, 2008). This means of analysis

should be considered to further highlight the potential of essential oils for antimicrobial purposes as well as augment their use in traditional practice.

8.3.5. Antimicrobial activity of carrier oils

Carrier oils are commonly employed in the field of aromatherapy for the topical application of essential oils. Carrier oils such as avocado oil, are believed to enhance the rate of essential oil absorption through the skin as well as act synergistically alongside essential oils for antimicrobial purposes (Clarke, 2008). As carrier oils are predominantly applied in combination with essential oils during aromatherapy, it is recommended that the antimicrobial potential of these be investigated independently and in combination with essential oils to determine possible interactions.

8.3.6. *In vivo* analysis

Aromatherapy is a practice in which the use of essential oils for antimicrobial purposes is undertaken without very stringent levels of authority or control (Price and Price, 1992). As essential oils have proven efficacy against certain antimicrobial agents *in vitro*, it seems feasable to determine the efficacy of the combinations examined in this study in whole body systems in order to validate their use.

Previous *in vivo* combination studies include that in which the antifungal (ringworm) effect of the essential oil combination *Cymbopogon martini* and *Chenopodium ambrosioides* was evaluated using a guinea pig model. It was determined that complete fungal death occurred after 7-21 days (Prasad *et al*., 2010). Another study conducted by Bassett *et al*. (1990) examined the antimicrobial effect of a 5% *M. alternifolia* oil solution on 124 patients suffering from acne. This essential oil solution was compared against a 5% benzoyl peroxide solution. From the clinical trial it was determined that the 5% *M. alternifolia* solution demonstrated similar effects to that of the control with less side effects experienced. A later clinical trial performed by Tong *et al*. (1992) further sought to determine the antimicrobial effects of a 10% *M. alternifolia* essential oil solution on 104 patients suffering with

Tinea pedis infections. According to Tong *et al*. (1992), the 10% *M. alternifolia* essential oil solution resulted in an improvement in symptoms but did not inhibit or eradicate the infection.

8.4. Conclusion

The antimicrobial study of *L. angustifolia* in combination with commonly blended essential oils, not only confirms antimicrobial potential, but also addresses a large void in scientific research. Essential oil use for therapeutic purposes has a rich and deep history seated in the use of multiple variances in combinations. This study aimed to identify the antimicrobial effects of these essential oil combinations to further augment their use in traditional practice. In so doing, it was noted that essential oils have antimicrobial potential independently and in 1:1 combinations and thus the objectives of the study were acheived. The antimicrobial potential of *L. angustifolia* in combination with conventional antimicrobial agents shows promise for future use as the oil potentiated the effects of 50% of the agents investigated. Therefore, the essential oil *Lavandula angustifolia* (Lavender) does exhibit synergistic antimicrobial activity when used in combination with other essential oils and conventional antimicrobial agents.

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APPENDIX A

A1. The 38th Annual Conference of the South African Association of Botanists (SAAB) 2012

The additive and synergistic antimicrobial effects of Frankincense and Myrrh – Essential oils from the predynastic period

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When investigating the number of publications on essential oils in combination, 94 461 journal articles have been published on essential oils of which only 6.5% are attributed to the effects of essential oils in combination. With this in mind, the aim of this study was to identify the antimicrobial properties of *Lavandula angustifolia* essential oil in combination with its identified essential oil combinations in order to identify possible synergistic interactions. Upon investigation of the possible essential oil combinations including *L. angustifolia*, 44 were identified. These essential oils were then collected and tested in combination with *L. angustifolia* essential oil against three micro-organisms, namely *Candida albicans* (yeast), *Staphylococcus aureus* (gram-positive bacteria) and *Pseudomonas aeruginosa* (gram-negative bacteria). Of the essential oil combinations, 75.0% possessed synergistic or additive antimicrobial activity. Four combinations were identified as synergistic when tested against two of the three micro-organisms at equal concentrations. These combinations include *Lavandula angustifolia* in combination with *Darcus carota* (ΣFIC 0.50); *Lavandula angustifolia* in combination with *Juniperus virginiana* (ΣFIC 0.29); *Lavandula angustifolia* in combination with *Cinamomum zeylanicum* (ΣFIC 0.40) and *Lavandula angustifolia* in combination with *Citrus sinesis* (ΣFIC 0.42). Isobolograms were then created from data obtained on the combinations antimicrobial activity at varying concentrations. Two of the four combinations, *Lavandula angustifolia* in combination with *Cinamomum zeylanicum* and *Lavandula angustifolia* in combination with *Citrus sinesis*, were identified as the most promising in synergy and thus their chemical nature was investigated for further analysis. The major chemical constituents of each essential oil were tested against the micro-organisms *C. albicans* and *S. aureus* at equal and varying concentrations to identify their role in the antimicrobial effect of the essential oil. Upon investigation it was identified that *C. zeylanicum* essential oil had an antagonistic effect in the essential oil combination with the same activity identified by its major constituent. The essential oil *C. sinesis* had a synergistic effect in the essential oil combination with the same activity identified by its major constituent.

A2. 33rd Annual Conference of the Academy of Pharmaceutical Sciences of South Africa (APSSA) 2012

The antimicrobial activity of *Lavandula angustifolia* essential oil in combination with other aroma-therapeutic oils.

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Purpose:
Essential oils are not only used singularly but have been used in combination for many years. There is, however, very little scientific evidence to support the claims made for combined antimicrobial efficacy. With this in mind, a study was designed to assess the antimicrobial activity of Lavender (*Lavandula angustifolia*) essential oil, in combination with other essential oils with antimicrobial relevance.

Methods:
Lavandula angustifolia and 44 other essential oils were tested in combination against *Candida albicans* (ATCC 10231), *Staphylococcus aureus* (ATCC 6538) and *Pseudomonas aeruginosa* (ATCC 27858). The antimicrobial activities were investigated using the micro-titre plate MIC method. Promising essential oil combinations demonstrating synergistic interactions were investigated further to identify the effect at various ratios. GC-MS analysis was undertaken to determine the composition of the oils.

Results:
Of the essential oil combinations studied, 76% demonstrated synergistic or additive antimicrobial effects when placed in combination with *L. angustifolia*. Four 1:1 combinations were synergistic when tested against two of the three micro-organisms. These combinations include *Lavandula angustifolia* in combination with *Daucus carota* (ΣFIC 0.50); *Juniperus virginiana* (ΣFIC 0.29); *Cinnamomum zeylanicum* (ΣFIC 0.40) and *Citrus sinensis* (ΣFIC 0.42). Two of the four combinations (*Lavandula angustifolia* in combination with either *Cinnamomum zeylanicum* or *Citrus sinensis*), were identified as the most promising in synergy in varying ratios, and thus the major chemical constituents of the essential oils were investigated further. It was identified that the antimicrobial effects of the essential oils is attributed to the major chemical constituent.

Conclusion:
Of the 44 combinations tested, only three essential oil combinations were identified as antagonistic. This suggests that the use of essential oils in combination is mostly favourable and validates the possible combined use in aromatherapy for antimicrobial purposes. Further *in vivo* studies are encouraged to validate this.

APPENDIX B

Table B.1 Antimicrobial essential oil combinations identified during literature review.*

Essential Oil	Amyris	Angelica	Basil	Bay	Benzoin	Bergamot	Birch	Black Pepper	Cajaput	Calendula	Camphor	Caraway	Cardamom	Carrot Seed	Cederwood	Chamomile	Cinnamon	Citronella
Amyris					x													
Angelica			x		x											x		
Basil								x							x	x		x
Bay															x			
Benzoin						x												
Bergamot																x		
Birch									x				x			x		
Black Pepper					x										x			
Cajaput		x				x	x						x					
Calendula																		
Camphor			x						x							x		
Caraway			x	x	x								x			x		
Cardamom			x			x									x			
Carrot Seed						x												
Cederwood					x	x										x	x	
Chamomile		x			x	x												
Cinnamon			x		x								x					
Citronella						x			x						x			
Clove			x		x	x		x							x		x	x
Coriander						x		x									x	x
Cypress						x									x			
Dill						x												
Elemi													x					
Eucalyptus					x										x			
Fennel																		
Fir			x									x			x			
Frankincense			x			x		x							x		x	
Galbanum																		x
Garlic																		
Geranium		x	x	x	x	x		x						x	x			x
Ginger				x					x			x	x		x		x	
Grapefruit						x							x					
Ho Leaf																		
Hyssop		x																
Immortelle						x										x		

Table B.1 continued Antimicrobial essential oil combinations identified during literature review.*

Essential Oil	Clove	Coriander	Cypress	Dill	Elemi	Eucalyptus	Fennel	Fir	Frankincense	Galbanum	Garlic	Geranium	Ginger	Grapefruit	Ho Leaf	Hyssop	Immortelle	Jasmine
Amyris					x				x	x		x						x
Angelica												x				x		
Basil						x						x				x		
Bay		x				x							x					
Benzoin		x	x						x									x
Bergamot		x	x			x						x						x
Birch									x				x					
Black Pepper							x		x									
Cajaput	x					x						x				x	x	
Calendula																		
Camphor																		
Caraway		x			x				x	x		x	x					
Cardamom	x						x		x				x					
Carrot Seed																		
Cederwood			x						x			x						x
Chamomile												x						x
Cinnamon	x	x				x			x	x			x	x				
Citronella			x			x						x						
Clove						x								x				
Coriander			x						x	x		x	x					x
Cypress									x									
Dill		x	x									x						
Elemi									x	x		x	x					
Eucalyptus		x	x															
Fennel			x									x						
Fir									x									
Frankincense										x		x		x				
Galbanum					x				x			x	x					x
Garlic																		
Geranium									x					x				x
Ginger	x	x			x	x			x			x			x			
Grapefruit	x	x	x									x	x					
Ho Leaf													x					
Hyssop			x				x					x						
Immortelle									x			x						
Jasmine												x						
Juniper			x				x		x			x	x	x				

Table B.1 continued Antimicrobial essential oil combinations identified during literature review.*

Essential Oil	Juniper	Lavandin	Lavender	Lavender Spike	Lemon	Lemon Grass	Lime	Linden	Litsea	Mandarin	Marjoram	Melissa	Myrrh	Myrtle	Neroli	Niaouli	Nutmeg	Olibanum
Amyris			x									x						
Angelica			x		x					x								
Basil	x		x			x	x				x	x			x			
Bay	x		x		x						x							
Benzoin	x		x		x								x					
Bergamot	x		x		x						x	x	x		x			
Birch			x		x													
Black Pepper	x		x		x						x							
Cajaput	x		x		x					x				x		x	x	
Calendula																		
Camphor			x									x						
Caraway			x															
Cardamom					x										x			
Carrot Seed	x		x		x		x					x			x			
Cederwood	x		x		x			x					x		x			
Chamomile			x		x					x	x				x			
Cinnamon			x		x					x			x					
Citronella			x		x										x			
Clove			x		x	x											x	
Coriander					x							x			x			
Cypress	x		x		x					x	x							
Dill										x				x				
Elemi			x						x			x						
Eucalyptus	x		x		x	x					x	x						
Fennel	x		x		x													
Fir			x											x		x		
Frankincense			x							x		x	x		x			
Galbanum																		
Garlic																		
Geranium	x		x		x		x				x				x			
Ginger	x				x		x								x			
Grapefruit	x		x		x										x			
Ho Leaf					x													
Hyssop			x		x							x						
Immortelle			x							x								
Jasmine			x												x			
Juniper			x		x	x	x					x						

Table B.1 continued Antimicrobial essential oil combinations identified during literature review.*

Essential Oil	Orange	Origanum	Palmarosa	Patchouli	Pettitgrain	Peppermint	Pine	Rose	Rosemary	Rosewood	Sage	Sandalwood	Tagetes	Thyme	Tea-Tree	Vetivert	Violet	Ylang-Ylang
Amyris								x		x	x							x
Angelica				x							x	x				x		
Basil								x	x		x	x			x	x		
Bay	x							x	x					x				x
Benzoin	x				x			x				x						
Bergamot			x	x			x		x			x			x			x
Birch	x												x	x				
Black Pepper			x						x			x						
Cajaput							x	x	x	x		x		x				
Calendula																		
Camphor																		
Caraway	x									x								
Cardamom	x							x				x						x
Carrot Seed	x				x				x							x		
Cederwood								x	x	x		x				x		x
Chamomile			x	x				x			x	x						x
Cinnamon	x						x		x					x				x
Citronella	x				x	x	x				x				x			x
Clove	x		x			x			x					x	x			
Coriander	x				x		x				x	x						
Cypress	x						x				x	x						
Dill	x				x				x									
Elemi	x								x	x								
Eucalyptus						x	x		x					x	x			
Fennel	x					x		x	x		x	x						
Fir										x								
Frankincense	x			x			x	x				x				x		
Galbanum			x				x	x					x			x		x
Garlic																		
Geranium	x		x	x	x			x	x	x	x	x			x			
Ginger	x		x	x	x	x		x	x	x		x		x		x		
Grapefruit	x		x						x			x						
Ho Leaf			x			x								x				
Hyssop	x								x		x	x						x
Immortelle	x				x			x		x								
Jasmine								x			x	x						
Juniper	x		x				x		x		x	x			x	x		

Table B.1 continued Antimicrobial essential oil combinations identified during literature review.*

Essential Oil	Amyris	Angelica	Basil	Bay	Benzoin	Bergamot	Birch	Black Pepper	Cajaput	Calendula	Camphor	Chamomile	Clove	Coriander	Cypress	Dill	Elemi
Lavandin						x						x					
Lavender				x		x						x	x				
Lavender Spike						x						x					
Lemon					x							x					
Lemon Grass			x										x	x			
Lime		x				x		x									
Linden					x												
Litsea cubeba			x														
Mandarin												x	x				
Marjoram						x						x			x		
Melissa			x	x		x											
Myrrh					x								x		x		
Myrtle						x								x		x	
Neroli					x	x						x					
Niaouli			x											x			
Nutmeg								x					x	x	x		
Olibanum																	
Orange		x						x					x	x	x		
Origanum		x	x														
Palmarosa						x							x				
Patchouli						x		x					x				x
Pettitgrain						x						x					
Peppermint					x							x			x		
Pine													x		x		
Rose						x						x					
Rosemary			x			x		x					x		x		
Rosewood			x			x							x	x			
Sage				x		x								x			
Sandalwood			x		x	x		x				x			x		
Tarragon		x										x					
Thyme						x		x				x	x				
Tea-Tree												x	x		x		
Vetiver					x												
Violet					x												
Ylang-Ylang						x						x					

Table B.1 continued Antimicrobial essential oil combinations identified during literature review.*

Essential Oil	Eucalyptus	Fennel	Fir	Frankincense	Garlic	Geranium	Immortelle	Juniper	Lavandin	Lavender	Lavender Spike	Lemon	Lemon Grass	Linden	Litsea	Niaouli	Nutmeg
Lavandin						x	x					x					
Lavender						x		x				x					x
Lavender Spike						x	x					x					
Lemon	x	x		x		x		x		x				x			
Lemon Grass	x					x				x						x	
Lime						x				x				x			x
Linden										x							
Litsea cubeba						x				x							
Mandarin				x		x		x		x							
Marjoram	x			x		x		x		x							x
Melissa				x		x		x		x							
Myrrh	x			x		x				x		x					
Myrtle										x		x	x				
Neroli						x				x		x					
Niaouli		x		x				x		x		x					
Nutmeg				x								x					
Olibanum																	
Orange				x		x		x		x		x					x
Origanum		x				x							x				
Palmarosa				x		x		x		x		x	x				
Patchouli				x		x				x		x	x				
Pettitgrain						x				x							
Peppermint	x									x		x				x	
Pine	x							x		x		x				x	
Rose						x				x							
Rosemary				x		x				x			x				
Rosewood				x		x				x		x					
Sage				x		x		x		x							
Sandalwood				x		x		x		x		x					
Tarragon			x					x		x							
Thyme	x							x		x		x	x				
Tea-Tree	x					x		x		x		x	x				
Vetiver				x		x				x							
Violet				x						X							
Ylang-Ylang				x						X							

Table B.1 continued Antimicrobial essential oil combinations identified during literature review.*

Essential Oil	Orange	Origanum	Palmarosa	Patchouli	Pettitgrain	Peppermint	Pine	Rose	Rosemary	Rosewood	Sage	Sandalwood	Tarragon	Thyme	Tea-Tree	Vetivert	Violet
Lavandin	x										x						
Lavender	x		x	x			x	x	x		x			x	x		
Lavender spike	x																
Lemon	x		x			x		x				x		x	x		
Lemon Grass	x		x			x			x			x		x	x	x	
Lime			x					x	x		x						x
Linden			x					x								x	x
Litsea cubeba	x				x			x	x	x						x	
Mandarin										x	x						
Marjoram	x							x	x	x		x			x		
Melissa				x	x		x	x		x					x		x
Myrrh					x	x	x	x				x		x			
Myrtle									x	x				x	x		
Neroli								x	x		x						
Niaouli	x					x	x		x		x						
Nutmeg	x			x					x						x		
Olibanum																	
Orange			x		x			x	x	x	x			x	x		
Origanum							x		x					x			
Palmarosa	x				x	x		x		x	x	x		x	x		x
Patchouli	x						x	x		x	x	x					
Pettitgrain				x							x	x					
Peppermint							x		x			x		x			
Pine									x			x		x	x		
Rose				x							x	x					
Rosemary	x				x	x	x							x			
Rosewood			x	x	x			x	x			x				x	
Sage	x		x						x			x		x	x		
Sandalwood			x	x				x							x	x	
Tarragon							x			x	x					x	
Thyme	x		x				x		x		x				x		
Tea-Tree	x		x				x		x		x			x			
Vetiver				x	x			x		x	x	x					x
Violet	x							x				x				x	
Ylang-Ylang	x			x				x		x	x	x				x	

x indicates the combination of essential oils, shaded area indicates combinations involving Lavender (the essential oil of importance in this study), *Sellar, 1992; Lawless, 1995; Curtis, 1996; Shealy, 1998; Hili, 2001; Buckle, 2003; Lawrence, 2005.

APPENDIX C

Table C.1 An analysis of the essential oils indicated for use in combination with Lavender for antimicrobial effects.

	Common name*	Scientific name*	Description of plant*	Origin*	Aroma*	Antimicrobial properties
 www.gardnersworld.com	Angelica	*Angelica archangelica*	A herb with small broad pointed leaves and green-white flowers	north Africa	sweet, musky	Antiseptic properties, and is particularly effective in the treatment of topical fungal infections**
 www.rkessentialoil.com	Basil	*Ocimum basilicum*	A herb with broad, pointed leaves and purple-white flowers	Asia and the Pacific islands	very sweet and slightly spicy	Bactericidal and antiseptic properties** and is particularly effective against the micro-organisms *B. cereus, B. pertussis, S. cerevisiae, S. pombe* and *T. utilus* ***
 www.alibaba.com	Bay	*Laurus nobilis*	Bay leaves are long, glossy and leather in texture and the tree bears small yellow flowers and black berries	southern Europe	sweet and spicy	Has been identified as having antiseptic and bactericidal activity**
 www.harmonyhealthfood.com	Benzoin	*Styrax benzoin*	A tree that produces large green leaves and white flowers, while the essential oil is produced from the resinous sap that exudates from the tree bark	Java, Sumatra and Thailand	sweet aroma similar to that of vanilla	Antiseptic activity and is used in the treatment of mouth ulcers**
 www.cellulite.co.za	Black Pepper	*Piper nigrum*	A forest plant that grows like a vine and has broad dark green leaves with white flowers and red fruit	Singapore, India and Malaysia	sharp and spicy	Antiviral and bactericidal properties, and is particularly useful in the treatment of flu **
 www.shivaexportsindia.co	Caraway	*Carum carvi*	A herb that grows up to two feet in height and possess brown fruit and pink-white flowers	Northern Europe, Africa and Russia	sweet and slightly peppery	Believed to be beneficial in the treatment of bronchitis, acne and superficial wounds**. Caraway essential oil has also been found to demonstrate activity against *C. albicans* and *E. coli* ***
 www.shopmania.co.uk	Carrot Seed	*Daucus carota*	Carrot seed essential oil is obtained from the wild species of carrot that produces stalks with whitish-purple flowers	Europe	sweet	Beneficial in the treatment of bronchitis and flu**
 www.duke.edu	Cedarwood	*Juniperus virginiana*	A large tree with flat, dense leaves that produces yellow flowers.	North America	woody	Believed to be beneficial in the treatment of general skin infections, lice, bronchitis and acne**. Has also been found to demonstrate activity against *C. albicans, P. ovale* and *P. orbicular***

Table C.1 continued An analysis of the essential oils indicated for use in combination with Lavender for antimicrobial effects.

	Common name*	Scientific name*	Description of plant*	Origin*	Aroma*	Antimicrobial properties
 www.celtner.org.uk	Chamomile	*Anthemis nobilis*	A herb that grows up to 12 inches in height and possess white flowers with a yellow centre	Britain	apple-like	Is believed to demonstrate anti-infective, antiviral and bactericidal properties and is beneficial in the treatment of gum disease, acne, boils and carbuncles**.
 www.membranes.multimania.	Cinnamon	*Cinnamomum zeylanicum*	An evergreen tree that possesses light brown quills that can grow up to 30 feet	Indonesia, the East Indies, Java and Madagascar	spicy, sweet and musky	Is believed to demonstrate anti-infective, antiviral, antimicrobial and antiseptic properties; and is beneficial in the treatment of general skin infections and lice, has also been found to demonstrate activity against *B. cereus, C. albicans, Cornybacterium, E. coli, P. aeruginosa, Propionibacterium, S. aureus, S. cerevisiae, S. mutans, S. pombe* and *T. utilis***
 www.en.wikipedia.org	Citronella	*Cymbopogon nardus*	A grass that grows up to three feet in height and has long thin leaves	Sri Lanka, Java, Burma and Madagascar	sweet and lemony	Is believed to demonstrate antiseptic properties and is beneficial in the treatment of athletes foot**
 www.mdidea.com	Clove	*Eugenia caryophyllus*	An evergreen tree that grows up to 30 feet in height and possess redish-brown nail shaped buds	Molucca islands and Indonesia	strong spicy	Believed to demonstrate antiseptic and bactericidal activity and is beneficial in the treatment of fleas, bronchitis, flu, acne, athletes foot, ulcers and superficial wounds**, has also been found to demonstrate activity against *B. cereus, C. albicans, Cornybacterium, E. coli, P. aeruginosa, Propionibacterium, S. aureus, S. cerevisiae, S. mutans* and *T. utilus***

Table C.1 continued An analysis of the essential oils indicated for use in combination with Lavender for antimicrobial effects.

	Common name*	Scientific name*	Description of plant*	Origin*	Aroma*	Antimicrobial properties
 www.heav.org	Cypress	*Cupressus sempervirens*	A cone shaped tree that once cut never grows again	the Mediterranean region	woody and slightly spicy	Believed to be beneficial in the treatment of gum disease and bronchitis** and has also been found to demonstrate activity against *B. pertussis, C. albicans, S. cerevisiae, S. pombe* and *T. utilus****.
 www.flicker.com	Elemi	*Canarium luzonicum*	A tree from which the essential oil is produced from the resinous sap that exudates from the bark	the Philippines	citrus and slightly spicy	Is believed to be beneficial in the treatment of general skin infections and acne**
 www.horizonherbs.com	Eucalyptus	*Eucalyptus globulus*	A tree that grows up to 300 feet in height	Australia	sharp	Antifungal, antiviral, antiseptic and bactericidal activity and is beneficial in the treatment of throat infections, lice, malaria, bronchitis, flu, pneumonia and chicken pox**, has also been found to demonstrate activity against *B. megalenum, C. albicans, E. coli, S. aureus, S. cerevisiae, S. pombe, T. utilus, N. gonorrhoea* and *H. simplex****.
 www.bikitube.com	Fennel	*Foeniculum dulce*	A herb that grows up to five feet in height and possess feathery leaves and yellow flowers	the Mediterranean region	floral and slightly spicy	Is believed to demonstrate antimicrobial and antiseptic properties and is beneficial in the treatment of gum disease and UTI's.** Fennel essential oil has also been found to demonstrate activity against *C. albicans, S. cerevisiae, S. pombe* and *T. utilus* ***
 www.conifers.org	Fir	*Abies balsamea*	A tree that possesses long green needles and brown cones	America and Canada	balsamic	Is believed to demonstrate antiseptic properties and is beneficial in the treatment of UTI's and bronchitis**
 www.moroccanfood.about.com	Geranium	*Pelargonium odoratissimum*	A hedge plant that grows up to two feet in height and produces pointed leaves and pink flowers	France, Reunion, Spain, Morocco, Egypt and Italy	sweet similar to rose but with a minty overtone	Is believed to posses antiseptic properties and is beneficial in the treatment of throat infections, UTI's, dysentery, enteritis, mouth ulcers, lice, acne and ringworm**. The essential oil has also been found to demonstrate activity against *C. albicans S. cerevisiae, S. pyogens, S. pombe* and *T. utilus****

Table C.1 continued An analysis of the essential oils indicated for use in combination with Lavender for antimicrobial effects.

	Common name*	Scientific name*	Description of plant*	Origin*	Aroma*	Antimicrobial properties
 www.meyerlemontree.com	Grapefruit	*Citrus grandis*	A tree that produces glossy leaves, white flowers and yellow fruits	Israel, Brazil and America	sweet and sharp	Is believed to demonstrate antiseptic properties and is beneficial in the treatment of general skin infections, common colds, flu and acne**. Grapefruit essential oil has also been found to demonstrate activity against *C. albicans S. cerevisiae*, *S. pombe* and *T. utilus****.
 www.gardensablaze.com	Hyssop	*Hyssopus officinalis*	A herb that grows up to two feet in height and produces purple-blue flower tops	Germany, France and Italy	sweet	Is believed to demonstrate antiviral, antiseptic and bactericidal properties and is beneficial in the treatment of throat infection, bronchitis, flu and whooping cough**
 www.ilehtinen.net	Juniper	*Juniperus communis*	An evergreen shrub that grows up to six to thirty feet in height and produces needle like leaves, yellow flowers and black-blue berries	Hungary, France, Italy, Yugoslavia and Canada	slightly woody	Is known to demonstrate antiseptic and bactericidal activity and is beneficial in the treatment of acne, leuccorhoea and cystits.** Juniper essential oil has also been found to demonstrate activity against *Propionibacterium* and *S. cerevisiae****
 www.dgmaromatics.com	Lemon	*Citrus medica limonum*	A thorny, evergreen tree that produces shiny, oval leaves, white-pink flowers and yellow fruit	India	fresh citrus	Is believed to demonstrate antiseptic properties and is beneficial in the treatment of general skin infections, throat infections, gum disease, bronchitis, flu acne, athletes foot, boils and warts**. It has also been found to demonstrate activity against *D. pneumoniae*, *P. aeruginosa*, *S. aureus*, *S. cerevisiae*, *S. pyogens*, *T. mentagophytes*, *T. utilus* and *V. cholerae****
 www.flicknver.com	Lemongrass	*Cymbopogon citrates*	A grass that grows up to three feet in height	India	sweet and lemony	Is believed to demonstrate antiseptic properties and is beneficial in the treatment of enteritis, lice, ticks, acne and athletes foot.** Lemongrass essential oil has also been found to demonstrate activity against *Cornybacterium*, *D. pneumoniae*, *Propionibacterium* and *T. utilus****
 www.anandaapothecary.	Litsea cubeba	*Litsea cubeba*	A tree that produces fragrant leaves, yellow flowers and spicy fruits	China and Malaysia	sweet citrus and fruity	Is believed to be beneficial in the treatment of *C. albicans* related infections**

Table C.1 continued An analysis of the essential oils indicated for use in combination with Lavender for antimicrobial effects.

	Common name*	Scientific name*	Description of plant*	Origin*	Aroma*	Antimicrobial properties
 www.flowersmacropics.colormagicphotography.	Marjoram	*Origanum marjorana*	A herb that grows up to ten inches in height and produces small oval leaves and pink-white flowers	Egypt	warm and slightly spicy	Has antiviral and bactericidal properties and is beneficial in the treatment of bronchitis and flu, has also been found to demonstrate activity against *P. aeruginosa, S. pullonum, V. enterocolitica*[***]
 www.mdide.com	Myrrh	*Commiphora myrrha*	A shrub that grows up to nine feet in height, with the essential oil produced from the resinous sap that exudates from the tree bark	north Africa, Asia and Somalia	musky	Known to demonstrate antiseptic properties and is beneficial in the treatment of general skin infections, throat infections, gingivitis, pyorrhoea, spongy gums, oral thrush, mouth ulcers, bronchitis, common colds, glandular fever, laryngitis, athletes foot and ringworm.' has also been found to demonstrate activity against *C. albicans, E. coli* and *S. mutans*[***]
 www.gardencoachpictures.wordpress.com	Myrtle	*Myrtus communis*	A wild growing bush that produces green-blue leaves, white flowers and black fruit	north Africa and Iran	sweet	Is believed to demonstrate antiseptic properties and is beneficial in the treatment of throat infections, acne, cystitis and urethritis[**]
 www.grandir-nature.com	Niaouli	*Melaleuca viridiflora*	A large tree that produces yellow flowers	Australia	sweet	Has antiseptic and bacteriacdal activity and is beneficial in the treatment of throat infections, dysentery, enteritis, worm infections, bronchitis, flu, pneumonia, tuberculosis, whooping cough, acne, boils, ulcers, cystitis and UTI's [**]
 www.bridgat.com	Orange	*Citrus aurantium*	A fruit, where the peel is used to produce an essential oil	China and India	zesty, citrus	Is believed to demonstrate antiviral properties and is beneficial in the treatment of gum disease, bronchitis and flu, has also been found to be effective in the treatment of *C. albicans, S. cerevisiae, S. pombe* and *T. utilus*[***]
 www.healingscents.net	Palmarosa	*Cymbopogon martini*	A grass that produces long growing, slender leaves and produces terminal growing dark red flowers.	India	sweet and floral with a hint of rose	Is believed to demonstrate antiviral and antiseptic properties and is beneficial in the treatment of general skin infections, dysentery, enteritis and acne, has also been found to demonstrate activity against *C. albicans, E. coli, P. aeruginosa, S. aureus, S. cerevisiae, S. pombe, T. mentagophytes* and *T. utilus*[***]

Table C.1 continued An analysis of the essential oils indicated for use in combination with Lavender for antimicrobial effects.

	Common name*	Scientific name*	Description of plant*	Origin*	Aroma*	Antimicrobial properties
 www.secretspa.com	Patchouli	*Pogostemon patchouli*	A shrub that grows up to three feet in height and produces furry leaves and white-purple flowers	Unknown	sweet and spicy	Is believed to demonstrate antiviral, antiseptic and bactericidal properties and is beneficial in the treatment of general skin infections, acne and athletes foot**
 www.flowers.vg	Rosemary	*Rosmarinus officinalis*	A herb that grows up to three feet in height and produces long, thin leaves and blue-lilac flowers	Asia	strong herbal	Is believed to demonstrate antifungal, antiseptic and bactericidal activity and is beneficial in the treatment of lice, bronchitis, flu, whooping cough, acne and UTI's ** Rosemary essential oil has also been found to demonstrate activity against *B. cereus, B. pertussis, P. orbicular, P. ovale, S. cerevisiae, S. mutans* and *S. pombe****
 www.centreforhealthyaging.org	Sage	*Salvia sclarea*	A herb that grows up to two feet in height and produces green-purple leaves and blue flowers	the Mediterranean region	sharp herbal	Is believed to demonstrate bactericidal properties and is beneficial in the treatment of general skin infections, gingivitis, mouth ulcers, glandular fever, whooping cough, acne, boils and carbuncles.' has also been found to demonstrate activity against *A. calcoacetica, B. linens, C. albicans, C. sporogenes, E. coli, Moraxella spp., S. aureus, S. epidermidis, S. mutans, S. pombe, S. pyogens* and *T. utilus****
 www.en.wikipedia.org	Sandalwood	*Santalum album*	A large evergreen tree that grows up to 15 metres in height.	Mysore, India	sweet woody	Is believed to demonstrate antiseptic and bactericidal properties and is beneficial in the treatment of general skin infections, throat infections, bronchitis, acne, boils, cystitis and UTI's.' has also been found to demonstrate activity against *C. albicans*
 www.tribes.tribe.net	Tagetes	*Tagetes minuta*	A shrub that produces feathery leaves and bright orange flowers	Central America	sweet citrus	Effective for antiviral properties and is beneficial in the treatment of general skin infections and warts** Tagetes essential oil has also been found to demonstrate activity against *C. albicans, S. cerevisiae, S. pombe* and *T. utilus****

Table C.1 continued An analysis of the essential oils indicated for use in combination with Lavender for antimicrobial effects.

	Common name*	Scientific name*	Description of plant*	Origin*	Aroma*	Antimicrobial properties
 www.apps.rhs.org.uk	Tarragon	*Artemisia dracunculus*	A herb that grows up to three feet in height and produces green leaves and white-grey flowers	the Middle East	herby, spicy	Is believed to be beneficial in the treatment of general skin infections.** Tarragon essential oil has also been found to demonstrate activity against *S. aureus, P. aeruginosa, S. faecalis* and *V. enterocolitica****
 www.herbosophy.co.au	Thyme	*Thymus vulgaris*	A herb that grows up to eight inches in height and produces green-grey leaves and white or purple-pink flowers	southern Europe	sweet and strongly herbal	Is believed to demonstrate antibiotic, antiseptic, disinfectant and germicidal properties and is beneficial in the treatment of general skin infections, gum disease, lice, worm infections, bronchitis, laryngitis, pharyngitis, tonsillitis, whooping cough, abcessess, acne, boils, carbuncles, gonorrhoea, leuccorhoea, non specific urethritis, cystitis and pyelitis**, has shown activity against *B. pertussis, C. albicans, C. sporogenes, E. coli, Moraxella spp., P. aeruginosa, S. aureus, S. cerevisiae, S. enteritis, S. pombe, S. pyogens, T. mentagophytes* and *T. utilus****
 www.aromashow.en.alibaba.com	Tea-tree	*Melaleuca alternifolia*	A small tree that grows up to 20 feet in height	New South Wales	fresh and pungent	Has antibiotic, antifungal, antiviral, antiseptic, bactericidal and germicidal properties ansd is beneficial in the treatment of otitis media, throat infections, gingivitis, worm infections, bronchitis, glandular fever, flu, whooping cough, acne, athletes foot, boils, carbuncles, ringworm, warts, whitlows and vaginal thrush.**, has also been found to demonstrate activity against *B. pertussis, C. albicans, E. coli, S. cerevisiae, S. pombe, T. utilus* and *T. violaceum****

Table C.1 continued An analysis of the essential oils indicated for use in combination with Lavender for antimicrobial effects.

	Common name*	Scientific name*	Description of plant*	Origin*	Aroma*	Antimicrobial properties
 www.domaromatov.ru	Vetiver	*Andropogon muricatus*	A grass that produces long growing, tall, slender leaves.	Tahiti, Java and Haiti	smoky	Is believed to demonstrate antiseptic properties and is beneficial in the treatment of general skin infections and acne, has also been found to demonstrate activity against *C. albicans, S. cerevisiae, S. pombe, T. utilus*[***]
 www.pinecrestgarden guy.blogspot.com	Ylang-Ylang	*Canangia odorata*	A small tree that produces pink, mauve and yellow flowers	Seychelles, Mauritius, Tahiti and the Philippines	sweet floral	Is believed to demonstrate antiseptic properties and is beneficial in the treatment of acne[**]

[*]Sellar, 1992.

[**] Sellar, 1992; Lawless, 1995; Curtis, 1996; Shealy, 1998; Hili, 2001; Buckle, 2003; Lawrence, 2006.

[***] Buckle, 2003

APPENDIX D

Table D.1 The chemical composition of the essential oil *Cinnamonum zeylanicum*.

RT	Constituents	Percentage Abundance	RT	Constituents	Percentage Abundance
8.202	Decane	t.a.*	34.552	*α*-Humulene	0.57
9.082	*α*-Pinene	0.54	35.212	*α*-Terpineol	0.15
9.214	*α*-Thujene	t.a.	35.266	Viridiflorene	t.a.
10.800	Camphene	0.21	35.389	Borneol	t.a.
12.583	*β*-Pinene	0.22	36.043	*ϒ*-Patchoulene	t.a.
14.464	*δ*-3-Carene	t.a.	36.283	Benzyl acetate	0.23
15.252	*α*-Phellandrene	0.80	36.431	Elixene	t.a.
15.923	*α*-Terpinene	t.a.	37.019	*δ*-Cadinene	t.a.
16.798	Limonene	0.25	38.406	*cis*-Sabinol	t.a.
17.251	*β*-Phellandrene	0.54	39.496	*p*-Cymen-8-ol	t.a.
18.942	*ϒ*-Terpinene	t.a.	40.303	Benzyl alcohol	t.a.
19.239	*cis*-*β*-Ocimene	t.a.	40.391	Safrol	t.a.
20.104	*p*-Cymene	0.54	42.114	3-Phenyl propyl acetate	t.a.
20.579	Terpinolene	t.a.	43.360	Caryophyllene oxide	0.39
25.412	2-Butoxy-ethanol	t.a.	43.764	Eugenol methyl ester	0.10
28.706	Copaene	0.60	44.744	*trans*-Cinnamaldehyde	0.53
29.694	Camphor	t.a.	45.465	Globulol	t.a.
29.991	Benzaldehyde	t.a.	46.595	Spathulenol	0.10
30.441	Linalol	0.72	47.271	Cinnamyl acetate	0.85
32.309	*β*-Caryophyllene	3.44	**47.673**	**Eugenol**	**80.06**
32.453	*β*-Panasinsene	t.a.	49.629	Eugenyl acetate	4.62
32.592	Aromadendrene	t.a.	61.011	Benzyl benzoate	3.06
				Total	99.62

*t.a. = trace amounts, **bold** indicates major chemical constituents.

Table D.2 The chemical composition of the essential oil *Citrus sinensis*.

RT	Constituents	Percentage Abundance
8.279	Decane	0.23
9.193	*α*-Pinene	0.30
13.371	Sabinene	0.28
14.642	*σ*-3-Carene	0.10
15.379	Myrcene	1.70
17.180	**Limonene**	**95.37**
17.464	*β*-Phellandrene	0.24
21.090	*p*-Cymene	t.a.*
25.553	*n-O*ctanal	0.19
28.832	2-Butoxy-ethanol	t.a.
28.832	*α*-Copaene	t.a.
29.067	Decanol	0.24
30.414	*β*-Cubebene	t.a.
30.566	Linalool	0.56
35.343	*α*-Terpineol	t.a.
36.126	Valencene	t.a.
36.450	Citral	0.11
37.167	Carvone	t.a.
	Total	99.66

*t.a. = trace amounts, **bold** indicates major chemical constituents.

Table D.3 The chemical composition of the essential oil *Daucus carota*.

RT	Constituents	Percentage Abundance	RT	Constituents	Percentage Abundance
8.232	Decane	0.38	33.413	*epi-β*-Santalene	0.20
9.141	*α*-Pinene	3.39	33.678	Sesquisabinene A	4.06
9.254	*α*-Thujene	t.a.*	34.186	*β*-Sesquiphellandrene	4.59
10.836	Camphene	0.20	34.607	*α*-Caryophyllene	0.47
12.629	*β*-Pinene	0.61	34.724	*α*-Humulene	0.46
13.284	Sabinene	3.04	34.935	*trans-β*-Bergamotene	0.76
13.441	Thujα-2.4(10)-diene	t.a.	35.076	*α*-Longipinene	t.a.
15.245	Myrcene	1.00	35.820	Verbenone	0.14
15.965	*α*-Terpinene	t.a.	36.156	*β*-Bisabolene	5.29
16.846	Limonene	0.70	36.219	*α*-Seliene	0.16
17.287	*β-P*hellandrene	t.a.	36.546	Carvone	0.16
18.989	*ϒ*-Terpinene	0.30	36.929	Geranyl acetate	1.13
20.145	*p*-Cymene	0.68	37.069	*δ*-Cadinene	t.a.
20.623	Terpinolene	t.a.	37.449	Bisabolene	1.58
25.439	2-Butoxy-ethanol	0.11	37.509	*α*-Curcumene	0.11
26.851	*p*-Cymenene	t.a.	38.019	Myrtenol	t.a.
27.578	Sulcatol	t.a.	39.135	*cis*-Carveol	t.a.
27.600	*trans*-Sabinene hydrate	t.a.	39.222	Germacrene	0.10
28.761	Daucene	1.49	39.340	Geraniol	1.04
30.180	Calarene	0.13	39.532	*p-C*ymen-8-ol	t.a.
30.289	*ϒ*-muurolene	0.33	43.400	Caryophyllene oxide	3.55
30.475	Linalol	0.24	**43.414**	**Carotol**	**44.41**
31.305	*α*-Funebrene	1.49	44.269	6-epi-Cubenol	2.26
31.440	*α*-Santalene	0.57	46.468	Spathulenol	0.12
31.767	Borneol acetate	0.39	46.623	*α-B*isabolol	t.a.
31.858	*β*-Funebrene	2.39	48.635	*α*-Eudesmol	0.19
32.046	*β*-Elemene	t.a.	50.485	Daucol	0.64
32.379	*β*-Caryophyllene	5.69	50.675	*ϒ*-Eudesmol	0.38
32.888	*cis-α*-Bergamotene	0.26	54.687	*(Z)*-asarone	0.27
				Total	96.18

*t.a. = trace amounts, **bold** indicates major chemical constituents.

Table D.4 The chemical composition of the essential oil *Juniperus virginiana*.

RT	Constituents	Percentage Abundance	RT	Constituents	Percentage Abundance
8.282	Decane	t.a.*	35.519	Alaskene	0.17
29.365	*α*-Funebrene	0.76	35.752	Selina 4.11-diene	1.39
29.990	Isolongifolene	0.66	36.223	*ϒ*-Hamachalene	0.79
30.927	*α*-Longipinene	0.90	36.480	Cedrene V6	0.78
31.569	***α*-Cedrene**	**12.47**	36.692	*α*-Cuprenene	2.33
31.685	*β*-Cedrene	4.43	37.174	*δ*-Cadinene	0.23
31.819	*α*-Barbatene	0.19	37.614	*arc*- Curcurmene	0.46
32.520	*β*-Funebrene	6.42	37.953	*β*-Cupenene	0.70
33.378	**Thujopsene**	**29.82**	39.190	Cupanene	4.02
33.640	*allo*-Aromadendrene	0.19	39.357	Calamanene	t.a.
33.909	*α*-Hamachalene	t.a.	45.172	Cubenol	0.18
34.109	*β*-Barbatene	0.18	45.590	*Unknown*	2.40
34.335	Isocaryophyllene	0.22	**46.754**	**Cedrol**	**15.03**
34.705	*α*-Humulene	0.12	47.419	Widdrol	1.93
34.778	Acoradiene	0.41	48.141	*α*-Cadinol	0.11
35.183	*β*-Hamacholine	0.28	48.701	*β*-Bisabolol	0.48
35.391	Bicyclosesquiphellandrene	0.22		**Total**	88.49

Unknown: m/z (%) = 191 (100); 121 (62), 108 (53), 81 (49), 93 (46), 161 (36), 207 (35), 41 (33), 135 (30), 69 (28)

*t.a. = trace amounts, **bold** indicates major chemical constituents.