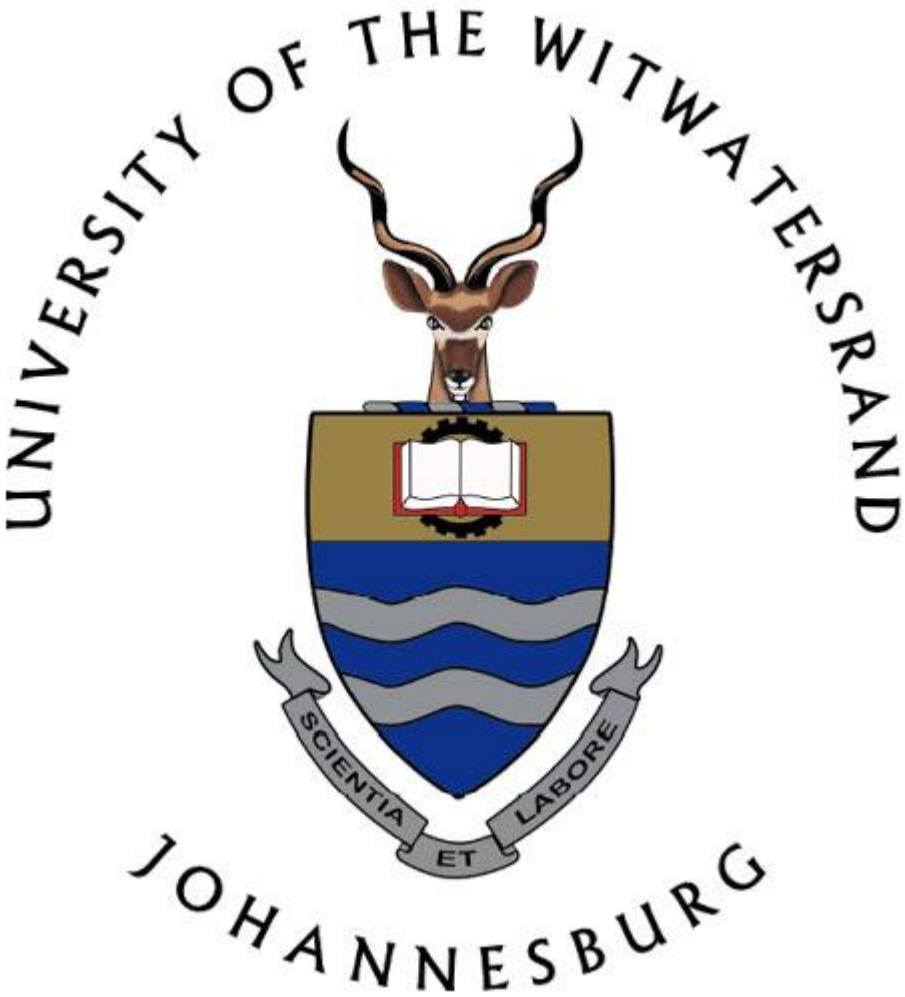
ANTIMICROBIAL ACTIVITY AND CHEMICAL ANALYSIS OF *EUCALYPTUS RADIATA* LEAF ESSENTIAL OIL



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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Pharmacy

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DECLARATION

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

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Gillian Dumsile Mahumane Date

DEDICATION

I would like to dedicate this work to, Georginah Thandi Mthimunye (Mommy), there are mothers, and then there is you. The strongest woman I have ever met and the most loving at the same time. You have had the biggest faith in me and have fought harder than I have ever fought to make sure that I stay up. When I am low I look at your life and think “wow, it can be done”. You have a fighting spirit and you never ever give up. You are absolute perfection, the best example for any young lady. You have always called us your angels, but mom, you are the angel.

PRESENTATIONS AND PUBLICATIONS ARISING FROM THIS STUDY

Presentations

- Mahumane, G.D., van Vuuren, S.F., Kamatou, G., Viljoen, A.M. and Kamatou, G. The potential for *Eucalyptus radiata* leaf essential oil use as a commercial antimicrobial. Podium presentation at the South African Association of Botanists (SAAB) 41st annual conference held at Tshipise Forever Resort, South Africa, 11 - 15 January 2015. (Refer to Appendix A for abstract).

- Mahumane, G.D., van Vuuren, S.F., Kamatou, G., Viljoen, A.M., Kamatou, G. and Ahmad, A. Therapeutic potential for the use of *E. radiata* oil as an antimicrobial. Oral presentation for the School of Therapeutic Sciences Biennial Research Day, University of the Witwatersrand (Faculty of Health Sciences), Johannesburg, South Africa., September 8, 2015. **Awarded best oral presentation for 2015** (Refer to Appendix B for abstract).

Publications

- Mahumane, G.D., van Vuuren, S.F., Kamatou, G., Sandasi, M. and Viljoen, A.M. (2016). Chemical composition and antimicrobial activity of South African *Eucalyptus radiata* leaf essential oil, sampled over a year. Submitted 27 January 2016 to the *Journal of Essential Oil Research*. (Refer to Appendix C for abstract).

ABSTRACT

Eucalyptus radiata is one of the understudied medicinal *Eucalyptus* essential oils. It is an important aromatic oil, used for a variety of infectious conditions independently or in combination with other essential oils. Its antiinfective uses are an indicator of the antimicrobial potential of the essential oil and its compounds. However, the chemical composition and antimicrobial activities of this plant growing in South Africa is yet to be investigated. A basis of scientific evidence needs to be established in order to better understand its therapeutic properties, antiinfective potential and advocate its proper use in medicinal practices. Therefore, this study was designed to determine yield, chemical composition, antimicrobial activity and antiquorum sensing activity of the *E. radiata* leaf essential oil. Another aspect was to determine the influence of seasonal variation and leaf age on the essential oil yield and chemical composition over a 12 month period.

Young and mature leaves of the *E. radiata* species were sampled monthly over a period of one year (January to December 2014). The essential oil was obtained using the hydro-distillation method. Higher yields were obtained in the seasons of summer and spring (0.90% - 4.31% w/w), characterized by high temperature and high rainfall in comparison to autumn and winter (0.14% - 2.83% w/w). The chemical composition was analysed by gas chromatography coupled to mass spectrometry (GC-MS). The major compounds identified within all samples regardless of seasonal variation and leaf maturity were; 1,8-cineole (64.1% ± 11.9), α-terpineol (12.4% ± 4.6) and limonene (3.6% ± 2.7).

The minimum inhibitory concentration (MIC) assay was used to determine the antimicrobial activity of the essential oil independently, in comparison to commercial *Eucalyptus* essential oils and in 1:1 combinations with other essential oils. The Streptococci (0.19 - 2.00 mg/ml) and *Lactobacillus acidophilus* (0.19 - 1.75 mg/ml) showed the highest sensitivities. The *E. radiata* sample exhibited similar antimicrobial efficacy to commercial *Eucalyptus* essential oils. The antimicrobial activities of the major compounds were evaluated independently and in combination at 1:1 ratios and in various ratios relative to the arrangement in the *E. radiata*

leaf essential oil. Independently, all major compounds; 1,8-cineole (2.00 mg/ml), α-terpineol (0.75 - 1.00 mg/ml) and *S*-(-)-limonene (0.25 - 0.75 mg/ml) and *R*-(+)-limonene (0.25 - 0.63 mg/ml) displayed noteworthy antimicrobial activity. The sum of the fractional inhibitory concentration (ΣFIC) was used to determine the type of interactions observed from the compound combinations. The 1:1 combinations resulted in more synergistic interactions in comparison to combinations at relative ratios. Combinations with limonene resulted in better antimicrobial activity.

When *E. radiata* essential oil was screened at 1:1 ratios with other oils, additive antimicrobial interactions were frequently demonstrated from the 1:1 combinations against *Staphylococcus aureus* (66.67%) in comparison to *Pseudomonas aeruginosa* (8.33%) and *Candida albicans* (16.67%).

The broth macrodilution assay was used to screen for antiantiquorumquorum sensing activity against the biomonitor strain *Chromobacterium violaceium* (ATCC 12742) in dependently and at 1:1 combinations. *Eucalyptus. radiata* leaf essential oil displayed antiquorum sensing activity against *C. violaceium* with a 95.30% percentage violacein inhibition at a minimum quorum sensing inhibitory concentration (MQSIC) of 0.50 mg/ml. The 1:1 combination of *E. radiata: Melaleuca alternifolia* resulted was the most noteworthy outcome, thus the major compounds were investigated further. Two synergistic interactions were noted with the 1:1 combinations of α-terpinene and α-terpineol (ΣFQSIC 0.19), and 1,8-cineole and α-terpineol (FQSICI 0.19).

This study demonstrated the *in vitro* antimicrobial properties of the *E. radiata* leaf essential oil, which may serve as credence for its use in the treatment of infectious conditions. The bioactivity of its major compounds highlights *E. radiata* leaf essential oil as a source of bioactive compounds with potential antimicrobial applications. This study also introduced *E. radiata* essential oil as a quorum sensing inhibitor.

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LIST OF ACRONYMS, SYMBOLS AND ABBREVIATIONS

% v/v: Percentage volume for volume

®: Registered trademark symbol

%: Percent

(Pty) Ltd: Proprietary limited company

±: Approximately

°C: Degree Celsius

ATCC: American type culture collection

B. cereus: *Bacillus cereus*

BC.: Before Christ

C. albicans: *Candida albicans*

C. neoformans : *Cryptococcus neoformans*

C. violaceum: *Chromobacterium violaceum*

CDC: Centers for Disease Control and Prevention

CLSI: Clinical and Laboratory Standards Institute

Compound ID: Compound identification

DMSO: Dimethyl sulfoxide

E. dives: *Eucalyptus dives*

E. camaldulensis: *Eucalyptus camaldulensis*

E. citriodora: Eucalyptus citrodora

E. coli: *Escherichia coli*

E. faecalis: *Enterococcus faecalis*

E. globulus: *Eucalyptus globulus*

E. radiata: *Eucalyptus radiata*

E. smithii: *Eucalyptus smithii*

FICI: Fractional inhibitory concentration

FQSICI: Fractional inhibitory concentration index

g: Gram

GC: Gas chromatography

h: hour

INT: *p*-iodonitrotetrazolium

K. pneumoniae: *Klebsiella pneumoniae*

L. acidophilus: *Lactobacillus acidophilus*

L. monocytogenes: *Listeria monocytogenes*

LB: Luria-Bertani

M. catarrhalis: *Moraxella catarrhalis*

MBC: minimum bactericidal concentration

mg/ml: milligram per milliliter

MIC: Minimum inhibitory concentration

min: minute

ml: Milliliter

mm: Millimeter

MQSIC: Minimum quorum sensing inhibitory concentration

MRSA: Methicillin-resistant *Staphylococcus aureus*

MS: Mass spectrometry

n.d: undated

n: Number of repetitions

NCCLS: National Committee for Clinical Laboratory Standards

nm: Nanometer

P. aeruginosa: *Pseudomonas aeruginosa*

QS: Quorum sensing

S. aureus: *Staphylococcus aureus*

S. mutans: *Streptococcus mutans*

S. pneumoniae: Streptococcus pneumoniae

S. pyogenes: Streptococcus pyogenes

S. sonnei: *Shigella sonnei*

S. typhimurium: *Salmonella typhimurium*

spp: Species

TM: Trademark symbol

TSA: Tryptone Soya agar

TSB: Tryptone Soya broth

v/v: Volume per volume

w/v: Weight per volume

w/w: Weight per weight

WHO: World Health Organization

µl: Microliter

CHAPTER 1
Introduction

1.1. Infectious conditions

As early as ancient Greek (430 - 427 BC) and Egyptian civilization (1157 BC) the negative impact of infectious diseases on human health has been documented throughout history (Brachman, 2003; Nelson and Williams, 2013; Littman, 2009). The spread of infectious diseases lead to the death of numerous populations without a known cause. It was not until the 1800's that the cause of infectious disease was identified. In 1876, Robert Koch presented the "Henle-Koch postulates", linking infectious diseases to micro-organisms (Nelson and Williams, 2013). The infectious disease to micro-organism link continued to hold merit in subsequent years.

1.1.1. Micro-organisms: an overview of trends in infectious conditions

Since 1876, numerous micro-organisms have been linked to various infectious diseases. These include bacteria, viruses, protozoa and fungi (Peterson, 1996; WHO, 2015). From a historical perspective, bacteria may be one of the oldest identified microbial causes of infectious disease. For example, reference to anthrax (caused by *Bacillus anthracis*) was noted in the book of Exodus (9:2-7) and may be regarded as the oldest infectious disease known (Williams and Barker, 2008). In addition, conditions documented to cause epidemics in ancient civilization (Greece and Egypt) included: leprosy (*Mycobacterium leprae*), tuberculosis (*Mycobacterium tuberculosis*) and diphtheria (*Corynebacterium diphtheriae*), which were also caused by bacteria (Nelson and Williams, 2013). Although not as detrimental as in earlier times, infectious conditions have remained a major healthcare problem as they are the second leading cause of death in the world (Fauci, 2001; Kolodziej, 2011). Lower respiratory infections, HIV/AIDS, diarrheal diseases, tuberculosis, malaria and measles, have been identified as the major contributors of mortality (Kolodziej, 2011). Respiratory infections are the most problematic of the infectious conditions to date and have remained one

of the top three leading causes of death in the world during the past decade. They claim approximately 4.25 million lives worldwide each year (CDC, 2015; WHO, 2015). Organisms associated with respiratory conditions include: *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Moraxella catarrhalis* and *Staphylococcus aureus*, which are also commensals of the respiratory tract (Wilmoth *et al.*, 2007). Following respiratory infections, diarrheal diseases are among the top ten causes of death in the world, reportedly killing 1.5 million people in 2012 (WHO, 2015). Diarrhoea is usually one of the symptoms of an infection of the gastrointestinal tract. Organisms associated with gastrointestinal conditions include: *Escherichia coli*, *Salmonella* spp., *Shigella* spp. and *Candida albicans* (oesophagitis), most commonly implicated in immunocompromised patients (Beeching *et al.*, 2011). It is interesting to note that, most of the pathogens implicated in these infectious conditions represent non-harmful, naturally occurring commensals of the body. However, under specific conditions (weakened immune system or altered microbiota) these micro-organisms become pathogenic and cause serious illnesses at which point they are referred to as opportunistic pathogens.

1.1.2. Management of infectious conditions

Medicinal plants, sanitation, nutrition and health education were among the preventative and control measures taken to manage infectious diseases, until the introduction of antibiotics in the 20th century (Brachman, 2003). The introduction of antibiotics in the 1940s introduced effective treatments for infectious conditions, thereby relieving the impact of infectious disease on human health (Kalia, 2013; Ling *et al.*, 2015). However, since then, incidents of microbial resistance to conventional therapies (antibiotics) have increased in recent decades (Warnke *et al.*, 2009; Ling *et al.*, 2015). The development of microbial resistance renders antimicrobial agents ineffective and threatens current therapies.

Inappropriate and overuse of antimicrobials is one of the contributory factors to the development of microbial resistance. Micro-organisms have developed various mechanisms of resistance. Mostly through the selection and exchange of genetic components, as a result of the selective pressure promoted by the overuse of antimicrobials (Davies and Davies, 2010). The most problematic bacteria identified recently are the extended-spectrum β-lactamase-

producing *E. coli* (ESBL-EC) and *Klebsiella pneumoniae* (ESBL-KP), carbapenem-resistant Enterobacteriaceae, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii,* hospital-acquired methicillin-resistant *S. aureus* (MRSA), and vancomycin resistant *Enterococcus* (VRE) (Marasini *et al.*, 2015). Other problematic infections include nosocomial (hospital-linked) infections with, *Enterococcus faecalis, E. coli*, *K. pneumoniae*, *P. aeruginosa*, *Salmonella* spp., *Serratia* spp., *S. aureus*, and *S. pneumoniae* (Davies and Davies, 2010). These represent some of the micro-organisms found within the normal human flora that have developed resistance to antibiotics. Antimicrobial resistance poses a number of concerns for healthcare such as treatment failure, prolonged therapy (chronic infections) and increased healthcare costs due to prolonged therapy, preparation of isolation rooms for quarantine of patients, sterilization of facilities (Tenover, 2006; Warnke *et al.*, 2009; Ling *et al.*, 2015; Marasini *et al.*, 2015). However, the biggest threat is the anticipated therapeutic dead-end. This is because; the rate at which microbial resistance to antibiotics is developing, currently exceeds the rate at which new antibiotics are being developed (Ling *et al.*, 2015). This therefore warrants, and has promoted the search for alternatives that can be used to manage these microbial infections (Marasini *et al.*, 2015).

1.2. Plants as alternative sources of antimicrobial agents

Hippocrates, the father of medicine (431 BC), stated "Let thy food be thy medicine and medicine thy food". In this quote, Hippocrates shows recognition of the potential of food for good health. Today, plants are well-known sources of nutrients e.g. vitamin C found in the fruit *Citrus sinensis* (orange) and phytochemicals (e.g. morphine found in *Papaver somniferum* (opium poppy) plant). Throughout the course of history, plants have been used for the management of infectious conditions, even prior to the knowledge of microbial cause (Lang and Bachbauer, 2011). For example, the Australian Aborigines would use *Eucalyptus* leaves to dress wounds in order to prevent infection of the wound. And a more popular example would be the use of *Vaccinium macrocarpon* (cranberry) juice for the treatment and prophylaxis of urinary tract infections, which has been well promoted by physicians and the public for years (Lynch, 2004; Rios and Recio, 2005).

The use of plants for therapeutic purposes continues today with increasing interest. Approximately 80% of people in developing countries rely on complementary and alternative

medicine (Shah *et al.,* 2012; Perumal Samy *et al.,* 2013). This is in part due to the aforementioned rise of microbial resistance to antibiotics. Other contributing factors include; the aforementioned history of use, therapeutic and cost effectiveness offered by these natural medicines, accessibility of plant products, increased patient interest in autonomy over medical care, and the adverse effects associated with the use of synthetic drugs (Cowan, 1999; Carson *et al.,* 2006; Shah *et al.,* 2012; Yap *et al.,* 2014).

1.2.1. Antimicrobial properties of plant products

From an antimicrobial perspective, one of the significant factors contributing to the focus on plants is that plants live in nature. These are environments having very high microbial loads, yet plants still manage to thrive (Chenia, 2013; Hossain *et al.*, 2014). This is an indicator that there may be some evolutionary adaptations or something plants produce that is able to manage or control colonization by these micro-organisms. Plants produce secondary metabolites as a defence mechanism against potential threats, such as micro-organisms, environmental stress and herbivores (Cowan, 1999; Ahmad *et al.*, 2014b). The ability of plants to continue to thrive in a high microbial loaded environment suggests that these phytochemicals may possess bacteriostatic, bactericidal, anti-virulence or anti-pathogenic properties. Of all plant products, essential oils are regarded as one of the most active principles obtained from plants, and have generally had the greatest application in the management of infectious conditions (Rios and Recio, 2005).

1.3. Essential oils

1.3.1. Therapeutic uses of essential oils

The practice of essential oils for therapeutic and antimicrobial effects has been recognized since ancient civilization (Burt, 2004; Lang and Bachbauer, 2011; Lawless, 2013; Ali *et al.*, 2015). For example, ancient Egyptians preserved mummies using essential oils and Chinese civilization have used aromatherapy as a complementary and alternative therapy for at least 60 000 years (Pearlstine, 2011; Ali *et al.*, 2015).

Nowadays, essential oils are among the three main complementary alternative medicines (CAM) used in the prevention/treatment of infectious conditions, and their use is steadily increasing (Wilkinson, 2005; Raut and Karuppayil, 2014; Ali *et al.*, 2015). Essential oils form the basis of aromatherapy, where they are usually administered in small quantities as they are highly concentrated liquids (Ali *et al.*, 2015). They can be administered via inhalation, used as baths, compresses, or applied topically on the skin, but are rarely taken orally (Ali *et al.*, 2015).

Essential oils and their compounds possess a wide range of therapeutic properties e.g. antimicrobial, anti-oxidant, antifungal and expectorant properties (Kovac, 2011; Lang and Bachbauer, 2011; Seow *et al.*, 2014). Due to their multipurpose nature, and antimicrobial potential, essential oils have numerous applications in medicine, aromatherapy, pharmaceutical products, medicinal supplements and nutraceutical companies (Lang and Bachbauer, 2011; Raut and Karuppayil, 2014). These products range from mouth rinses (Listerine® mouthwash), disinfectants (Domestos® Lavender Blast Thick Bleach) to washing powders (Surf Essential Oils Powder®). Essential oils have also been used as food additives for their preservative function (Hyldgaard *et al.*, 2012; Tongnuanchan and Benjakul, 2014).

Essential oils from *Eucalyptus globulus* (*eucalyptus*), *Mentha piperita* (peppermint), *Citrus sinensis* (orange), *Mentha arvensis* (cornmint) and *Citrus limon* (lemon) are the principle essential oils produced for industrial purposes (Djilani and Dicko, 2012). *Santalum album* (sandalwood), *E. globulus (eucalyptus*), *Pelargonium graveolens* (geranium), *Lavandula officinalis* (lavender), *Boswellia carteri* (frankincense), *Rosa damascena* (rose), *M. piperita* (peppermint), *Citrus limon* (lemon), *Rosmarinus officinalis* (rosemary), *Melaleuca alternifolia* (tea tree) and *Jasminum officinalis* (jasmine) essential oils are the principle essential oils used for domestic purposes (Djilani and Dicko, 2012).

1.3.2. Properties of essential oils

Essential oils are volatile oily liquids obtained from various plant parts such as leaves (eg *Eucalyptus citriodora*), flowers (*L. officinalis*), seeds (e.g. *Piper nigrum*), twigs, bark, fruits (*Citrus spp*.) and roots (e.g. *Vetiveria zizanioides*) amongst others (Burt, 2004; Bakkali *et al.*,

2008; Başer and Buchbauer, 2010; Seow *et al.*, 2014; Tongnuanchan and Benjakul, 2014; Perricone *et al.*, 2015). Essential oils are regarded as the "chemical weapons" of plants, with various functions such as: the protection of plants (antimicrobial and antifungal properties) or to repel or attract certain animals (odour) (Bakkali *et al.*, 2008; Hamid *et al.*, 2011; Pearlstine, 2011).

1.3.4. Essential oil extraction

Documented as early as the 9th century, distillation is one of the oldest methods employed to obtain essential oil (Burt, 2004). Throughout history, distillation remained a relevant method. To date, hydro/steam distillation are the most commonly used methods to obtain essential oils (Burt, 2004; Seow *et al.*, 2014; Amenaghawon *et al.*, 2014; Perricone *et al.*, 2015). According to Amenaghawon *et al.* (2014), these distillation-based methods are preferred as they avoid decomposition of the essential oils and allow for operation with small volumes. The amount of essential oil that can be obtained varies due to a number of interrelated factors. These include: the growth stage of the plant, seasonal or geographical growth conditions, plants species, harvest time or plant part distilled (Hamid *et al.*, 2011).

1.3.5. Chemical composition

Gas chromatography and mass spectrometry are methods commonly used in combination to determine the chemical composition of essential oils (Lang and Bachbauer, 2011). Gas chromatography allows for separation of the compounds from the essential oil mixture. Whereas the mass spectrometer aids in the identification and quantification of the compounds within the essential oil (Hamid *et al.*, 2011). These methods have allowed for great progress in the chemical analysis of numerous essential oils (Pitarević, 1984; Verma *et al.*, 2011; Burt, 2004).

Essential oils have been identified as highly concentrated, complex mixtures comprising of a wide range (approximately 20 - 60) of volatile compounds (Tongnuanchan and Benjakul, 2014; Yap *et al.*, 2014). These compounds generally represent saturated and unsaturated hydrocarbons, alcohols, aldehydes, esters, ethers, ketones, oxides phenols and terpenes

(Tongnuanchan and Benjakul, 2014; Ali *et al.*, 2015). In an essential oil, these compounds appear in unique ratios. Generally, between 1 - 3 compounds represent a large ratio of the oil composition (20 - 70%) (major compounds), while other compounds represent a smaller (and even trace amounts) ratio of the oil composition (minor compounds) (Yap *et al.*, 2014). The unique ratio at which these compounds occur is known as the 'chemical composition' of the essential oil (Nasser AL-Jabri and Hossain, 2014). This composition varies between essential oils of different plant sources. For example, the main component of *Eucalyptus maideni* is 1,8-cineole (83.59%) while D-limonene (75.07%) is the main component in *Citrus paradisi* (grape fruit) peel (Okunowo *et al.*, 2013; Sebei *et al.*, 2015).

The ratio at which compounds appear in an essential oil is not fixed. The chemical composition of an essential oil may vary depending on a number of factors i.e. geographical origin, environmental growth conditions (soil type, water availability, temperature), harvest time (leaf age) or distillation process, genetic composition of plant species (Sartorelli *et al.*, 2007; Bendaoud *et al.*, 2009; Djilani and Dicko, 2012). The results of these variations are known as the chemotypes. The same plant species can produce more than one type of essential oil. For example, a number of *Eucalytpus* species produce more than one chemotype. These include *Eucalyptus camaldulensis*, *E. citriodora*, *Eucalyptus dives* and *Eucalyptus radiata* with five, four, five and six chemotypes respectively (Pearson, 1993; Moudachirou *et al.*, 1999; Coppen, 2002). An example of the chemotypes of *E. radiata* leaf essential oil is shown is Table 1.1. The presence of chemotypes presents a challenge with regards to predicting the essential oil quality, and properties which crucially impacts the market value of the essential oil (Raut and Karuppayil, 2014).

Table 1.1. Percentage composition ranges of four key compounds of the six chemotypes of *E. radiata* leaf essential oil (Coppen, 2002).

Chemotype	**Compund (%)**			
	1,8-Cineole	**α-Phellandrene**	**Piperitone**	**Terpinen-4-ol**
1	2.00 - 12.00	4.00 - 27.00	21.00 - 55.00	2.00 - 26.00
2	7.00 - 27.00	300 - 33.00	0.80 - 10.50	2.00 - 37.00
3	4.00 - 27.00	3.00 - 23.00	0.00 - 19.00	12.00 - 36.00
4	9.00 - 23.00	1.00 - 7.00	1.00 - 6.00	14.00 - 28.00
5	30.00 - 60 00	3.00 -20.00	0.00 - 6.00	2.00 - 23.00
6	58.00 - 76.00	1.00 - 20.00	1.00 - 3.50	3.1 - 6.00

1.3.6. Antimicrobial properties of essential oils: the science

The antiseptic properties of essential oils have been known since antiquity, however according to Dorman and Deans (2000), attempts to typify and validate the antimicrobial properties in laboratory settings can be dated to the early 1900's. To date, the minimum inhibitory concentration (MIC) (a dilution method) assay is cited by most researchers and is the preferred method for antimicrobial evaluation of plant samples and essential oils (Eloff, 1998; Burt, 2004; Lang and Bachbauer, 2011; van Vuuren *et al.*, 2014).

There is mounting *in vitro* evidence demonstrating the antimicrobial properties of essential oils, against a broad-spectrum of pathogenic micro-organisms associated with food spoiling, wound, dental and respiratory related pathogens for example (Dorman and Deans, 2000; Burt, 2004; Edris, 2007; Hamid *et al.*, 2011; Hammer and Carson, 2011; Lang and Bachbauer, 2011; Andrade *et al.*, 2014; Raut and Karuppayil, 2014; Yap *et al.*, 2014; Ali *et al.*, 2015; Dagli *et al.*, 2015; Freires *et al.*, 2015). It is clear from previous studies that essential oils indeed possess antimicrobial activity, and have potential for application in the food and pharmaceutical industries as sources of antimicrobial alternatives.

Modes of antimicrobial action of essential oils and structure-activity relationships

The mode of antimicrobial action of essential oils is a complex concept. It is a challenge to elucidate specific mechanisms of action due to the variable nature of essential oils and their chemical composition. However, several observations have been made in this ongoing research area;

Concentration dependent activity: The antimicrobial activity of an essential oil is dependent on the concentration of the essential oil, with activity increasing as the concentration increases. These results are usually reported as the minimum inhibitory concentration (MIC). An MIC is the lowest concentration of essential oil required to inhibit the growth of the micro-organism. This effective concentration (MIC) varies between essential oils, whereby some essential oils exhibit antimicrobial activity at lower concentrations in comparison to others. For example, *Eucalyptus aetheroleum* and *Rosmarini aetheroleum* exhibited minimum

inhibitory concentrations (MIC) of 0.390% and 0.195% v/v respectively against *S. aureus* (Bosnic *et al.*, 2006). The lower the MIC, the better the antimicrobial activity of the essential oil. According to literature, the essential oils of *Thymus vulgaris* (thyme) and *Origanum vulgare* (oregano), *Cymbopogon citratus* (lemongrass), *Aniba rosaeodora* (rosewood), *Syzigium aromaticum* (clove) oils are among the most active essential oils (Burt, 2004; Raut and Karuppayil, 2014).

Differential activity against various micro-organisms: Essential oils exhibit differential activity against various micro-organisms. For example *Eucalyptus aetheroleum* exhibited MIC's of 0.390% and 0.097% v/v against *Staphylococcus aureus* and *Bacillus subtilis* respectively (Bosnic *et al.*, 2006). A number of literature reviews have reported on the trend that Gram-negative micro-organisms are generally less susceptible to the effects of essential oils in comparison to Gram-positive bacteria (Burt, 2004; Trombetta *et al.*, 2005; Boire *et al.*, 2013; Nazzaro *et al.*, 2013; Yap *et al.*, 2014). *Pseudomonas aeruginosa* in particular, has been noted to generally be the least susceptible to the inhibitory effects of essential oils (Raut and Karuppayil, 2014). However, this generalization is not concrete and the susceptibility of Gram-negative bacteria is subject to variation due to genus and species (Burt, 2004; Boire *et al.*, 2013; Yap *et al.*, 2014). For example Bosnic *et al.* (2006) reported an MIC of 0.390% for *Eucalyptus aetheroleum* against both *Staphylococcus aureus* (Gram-positive) and *Pseudomonas aeruginosa* (Gram-negative). Both Gram-positive and Gram-negative pathogens showed the same susceptibility to the essential oil (Bosnic *et al.*, 2006).

Bioactive compounds: Previous studies confirm that individual essential oil compounds possess varied antimicrobial activity. Some compounds exhibit higher activities than others. It has been noted that essential oils containing phenolic compounds such as carvacrol, thymol and eugenol, generally possess strong antimicrobial activities (Burt, 2004; Dorman and Deans, 2000; Boire *et al.*, 2013; Saad *et al.*, 2013; Raut and Karuppayil, 2014; Yap *et al.*, 2014). Phenolic compounds work by cell membrane disruption, therefore, the mechanisms of action of the essential oils are assumed similar to that of the individual compounds. Apart from the type of compound present, stereochemistry has been noted to affect antimicrobial activity of essential oils (Saad *et al.*, 2013; van Vuuren and Viljoen, 2007).

Since essential oils are complex mixtures of various compounds, there is ongoing research to try and elucidate possible structure-activity relationships. One of the major research areas has been in evaluating the relationship between the antimicrobial properties of essential oils and their chemical composition.

Crude essential oil versus major compounds: There is an ongoing speculation in literature as to whether the antimicrobial activity of an essential oil is due to the activity of their major compounds or the interaction between major and minor compounds in combination. Major compounds are known to have a determinant role in the biological properties of some essential oils (Freires *et al.,* 2015). Li *et al.* (2014) showed that the essential oil of *Cinnamomum longepaniculatum* and its major compound 1,8-cineole both showed the same MIC values against *E. coli* (of 3.13 µl/ml), and *Staphylococcus aureus* (6.25 µl/ml). In comparison, the compounds present in lower amounts (α-terpineol and terpinen-4-ol) within *Cinnamomum longepaniculatum* essential oil showed lower MIC values against the same micro-organisms (Li *et al.*, 2014). In this instance, it can be argued that the strong correlation of the major compound to the essential oil, is due to the amplified effects of the major compound due to its high concentration. The effects of the minor compounds could be masked due to their low concentration.

Compound interactions: In the same note, whole essential oils may exhibit higher antimicrobial activity than accounted for by the additive effects of their major compounds (Bassole and Juliani, 2012; Vimal *et al.,* 2013). In this instance, it is argued that the antimicrobial properties of any essential oil are as a result of the synergistic effects of the interactions between major and minor compounds (Bassole and Juliani 2012; Yap *et al.*, 2014). There are four outcomes that can be achieved from compound interactions: synergy, additive, indifferent (non-interactive) or antagonistic interactions (van Vuuren and Viljoen, 2011). The outcome of compound interactions is influenced by the unique ratio of the compounds (van Vuuren and Viljoen, 2007; Burt, 2004; Seow *et al.*, 2014).

Essential oils in combination: In aromatherapy, essential oils are often used in combination (de Rapper *et al.,* 2013). This is in the belief that the combination will have a greater therapeutic outcome in comparison to individual essential oils. A combination of essential oils

results in an increased number of bioactive compounds, each with a unique therapeutic effect. Each essential oil may have more than one therapeutic property, ranging from antiinfective to anti-inflammatory properties (Kovac, 2011). Combination blends thus offer the combination of different properties. Theoretically, the various compounds within combinations can affect multiple target sites in the micro-organisms, resulting in greater antimicrobial effects (through e.g. sequential inhibition of biological pathways, similarity in mechanisms etc.). This ongoing hypothesis presents a new approach to increasing the efficacy of essential oils by taking advantage of their synergistic and additive effects. Mixtures of essential oils have proven to possess in some instances, synergistic effects when combined. An example would be the synergistic effect observed from the combination of *Lavandula angustifolia* and *Juniperus virginiana* essential oils. In combination, these essential oils displayed synergistic effects when tested against *Candida albicans* (de Rapper *et al.*, 2013). Synergistic interactions result in microbial inhibition at lower concentrations. The practical benefit of this is that lower concentrations of essential oils can be used. This would be beneficial as some essential oils have unfavourable scents or tastes. The practical implication of this, for example is in the food industry, where flavours and odours of essential oils may need to be masked (Bassole and Juliani, 2012). Synergistic combinations will allow less of that oil to be used, reducing that unwanted scent and possibly masking it with another more pleasant scent.

According to Higley and Higley (1998) essential oils are classified as either personifiers, enhancers, equalizers and modifiers depending on their function in the combination blend. With regards to synergy, the purpose of an enhancer is to enhance the properties of other oils in the blend (Higley and Higley, 1998). Compounds with similar functional groups are known to have synergistic effects. According to Rhind (2012), synergy is achieved when essential oils with similar functional groups are combined, this is known as horizontal synergy. However this hypothesis (‘horizontal synergy’) is yet to be validated. According to Bassole and Juliani (2012), compounds with similar structures tend to show additive rather than synergistic effects.

Antiquorum sensing activity: Micro-organisms release chemically diverse volatile compounds which can act as infochemicals facilitating communication among micro-organisms, between different types of micro-organisms and between micro-organisms and

their host (Schmidt *et al.*, 2015). An infochemical is a chemical that transmits information with the ability to induce biological and behavioural functions (Ararsa, 2014; Schmidt *et al.*, 2015). Understanding the function of these compounds in gene expression can aid in understanding possible ways to intercept virulence. Quorum sensing is a cell density dependent form of cell-to-cell communication, which plays a role in the regulation of these infochemicals (Schmidt *et al.*, 2015). The quorum sensing process enables the bacterial cells to produce, sense and respond to these infochemicals, signalling molecules known as autoinducers. These auto-inducers allow for communication between bacteria resulting in co-ordinated community behaviour (Koh *et al.*, 2013). These group behaviours lead to the success of many bacterial functions such as biofilm formation, release of toxins, sporulation, motility, conjugation and expression of virulent genes (Delalande *et al.*, 2005; Stauff and Bassler, 2011; Alvarez, 2012; Jaramillo-Colorado *et al.*, 2012; Koh *et al.*, 2013). Manipulation of this cell-density dependent regulation of gene expression (quorum sensing) could provide a useful and alternative approach to intercepting pathogenicity.

A number of opportunistic micro-organisms are dependent on the quorum sensing system for the expression of pathogenicity (Koh *et al.*, 2013). Micro-organisms identified to operate on an analogous cell density regulated system include *S. pneumoniae, B subtilis, S. aureus, Erwinia carotovora, E. stwearti, Enterobacter agglomerans, Serratia liquifaciens, Yersinia enterolitica, Agrobacterium tumefaciens, P. aeruginosa, P. aureofaceins* and *Rhizobium leguminosarum.* In these micro-organisms, the AHL-mediated signal system has been identified in contributing to the regulation of carbapenem and phenazine biosynthesis, plasmid conjugal transfer, swarming, cessation of growth, capsular polysaccharide synthesis, production of exoenzyme virulens determinants and cytotoxins in human and plant pathogens (McClean *et al.*, 1997; Defoirdt *et al.*, 2010; Eris and Ulusoy, 2013). This study will focus on *Chromobacterium violaceum* (ATCC 12472). Although C. *violaceum* infection is rare, this micro-organism may cause skin lesions, liver abscesses and septicaemia, which may be fatal (Sneath *et al.*, 1953; Martinez *et al.*, 2000). *Chromobacterium violaceum* is the most studied micro-organism in quorum sensing research. It is known to rely on the quorum sensing system for the expression of virulent genes (Stauff and Bassler, 2011). The quorum sensing system in *C. violaceum* consists of a CviI (auto-inducer synthase homologue) / CviR (cognate receptor homologue) circuit. This CviI/CviR circuit controls virulens (Stauff and Bassler,

2011). Bacteria (*C. violaceum*) consistently produce signalling molecules (auto-inducers). *Chromobacterium violaceum* produces and responds to cognate auto-inducer molecules, N-hexanoyl-L-acylhomoserine lactone (C6-AHL) and C4-AHL. At low cell density, the concentration of these autoinducers is too low resulting in unliganded receptors, however, as the cell density increases, the extracellular amount of these autoinducers increases. These accumulated acylated homoserine molecules bind to the CviR receptor. The result of this complex is the well-documented induction of the expression of violacein (purple pigment) production (Stauff and Bassler, 2011; Alvarez *et al*., 2012; Chenia, 2013; Koh *et al*., 2013). Figure 1.1 gives an overview of the proposed quorum sensing signalling system collaborated from literature. Inhibition of violacein production by a substance (for example, an essential oil) is used as a marker for antiquorum sensing activity (Eris and Ulusoy, 2013).

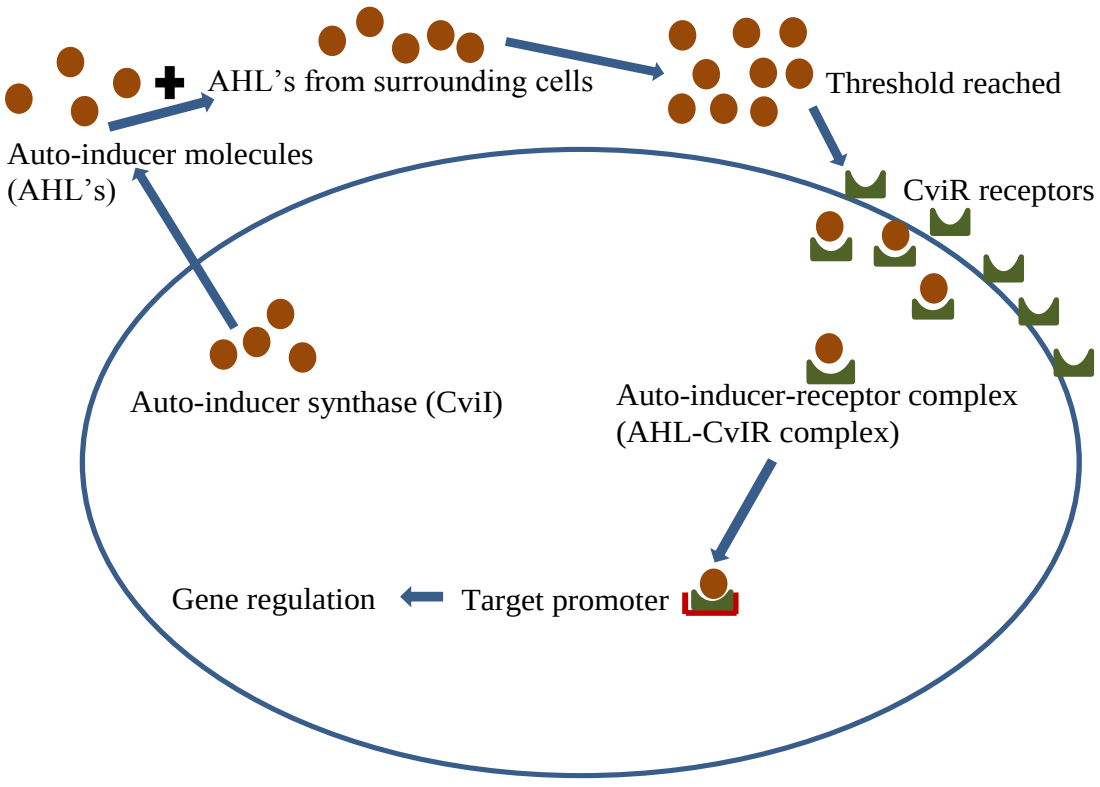

Figure 1.1. Diagram depicting the proposed quorum sensing signalling system of a *C. violaceum* ATCC 12472 cell used in this study. Autoinducer synthase (CviI) produces autoinducer molecules (AHL's) which are released out of the cell. Once the autoinducer threshold is reached, the autoinducers bind to their cognate receptor (CviR). The autoinducer-receptor complex binds to the appropriate target promoter ( ) triggering violacein production (Stauff and Bassler, 2011; Alvarez *et al.*, 2012; Chenia, 2013; Eris and Ulusoy, 2013; Koh *et al.*, 2013).

The antiquorum sensing activities of some essential oils and their compounds have been investigated (Khan *et al.*, 2009; Szabó *et al.*, 2010; Alvarez *et al.*, 2012; Jaramillo-Colorado *et al.*, 2012; Eris and Ulusoy, 2013; Kerekes *et al.*, 2013; Ahmad *et al.*, 2014b; Bai and Vittal, 2014; Luís *et al*., 2015.). In comparison to antimicrobial studies (inhibition of bacterial growth), less attention has been paid to the antiquorum sensing activities of essential oils, in particular *Eucalyptus* essential oils. Only four studies have reported on the antiquorum sensing activity of *Eucalyptus* essential oil (Khan *et al.*, 2009; Szabó *et al.*, 2010; Eris and Ulusoy, 2013; Luís *et al*., 2015). Khan *et al.* (2009) and Eris and Ulusoy (2013) found *E. globulus* to exhibit no antiquorum sensing activity against *C. violaceum*. In contrast, Szabó *et al.* (2010) noted moderate activity for *E. globulus* essential oil at 10% v/v and 3 mm zone of inhibition. While Luís *et al.*, 2015 noted both *Eucalyptus radiata* and *E. globulus* to exhibit antiquorum sensing activity with *E. radiata* presenting the best antiqourum sensing activity out of the two *Eucalyptus* essential oils. In three of the four studies, the chemical composition of the essential oils was not reported, making comparison of results difficult. More research needs to be done on *Eucalyptus* essential oils with regards to their antiquorum sensing properties.

Findings from these previous studies suggest that inhibition of quorum sensing may provide unexplored targets for the development of new antimicrobials (Khan *et al.*, 2009; Szabó *et al.*, 2010; Alvarez *et al.*, 2012; Jaramillo-Colorado *et al.*, 2012; Eris and Ulusoy, 2013; Kerekes *et al.*, 2013; Ahmad *et al.*, 2014b; Bai and Vittal, 2014; Luís *et al*., 2015). For example, unlike the conventional bactericidal and bacteriostatic mechanisms studied so far, with quorum sensing inhibition, the micro-organisms are neither killed nor is their growth inhibited, which in turn may avoid triggering the development of microbial resistance (van Vuuren and Viljoen, 2011; Chenia, 2013; Koh *et al.*, 2013).

1.4. *Eucalyptus* essential oil

Literature on essential oil research is vast and mounting. However, among the numerous essential oils studied, species belonging to the Myrtaceae family are most popular in terms of antimicrobial research (Burt, 2004; Raut and Karuppayil, 2014).

Due to their antimicrobial properties, essential oils from the Myrtaceae family are used in creams, soaps and toothpastes (Luqman *et al.*, 2008). These include the essential oils of *S. aromaticum* (Clove); *T. vulgaris* (thyme); *Thymus* spp.; *M. alternifolia* (tea tree); *E. globulus* (blue gum); *M. fragrans* (nutmeg). Therefore, further exploration of potential of the essential oils of the species of this family is warranted (Raut and Karuppayil, 2014; Hammer and Carson, 2011). In particular: commercialized, pharmaceutically relevant, medicinal essential oils such as *Eucalyptus* essential oil (Luqman *et al.*, 2008).

1.4.1. Uses and therapeutic relevance of *Eucalyptus* essential oils

Traditionally, *Eucalyptus* essential oil was used in Aboriginal medicine to heal wounds and fungal infections (Takahashi *et al.*, 2004; Sani *et al.*, 2014). Other than wound healing, *Eucalyptus* essential oil is claimed to be effective for the treatment of a variety conditions. These include infections of the genitourinary system (e.g. leucorrhea (whitish, yellowish discharge vaginal discharge resulting from vaginal infection) and cystitis); respiratory system (e.g. throat infections, bronchitis, sinusitis, coughs, bronchitis, asthma and sinusitis); skin conditions (acne, infected wounds, cuts, burns, herpes) dental conditions (mouth sores, gum disease and gingivitis) and a variety of other conditions associated with microbial infections (Derwich *et al.*, 2009; Lawless and Roche, 2002; Sani *et al.*, 2014; Ali *et al.*, 2015). The most common association of *Eucalyptus* oil is with respiratory relief (Luqman *et al.*, 2008).

Eucalyptus oil is a global source of medicinal essential oil with a range of traditional uses that have translated commercially. *Eucalyptus* essential oil is an ingredient of more than a 100 over-the-counter products, most of which were designed for use in the treatment of upper respiratory tract infections and are over the counter products designed for the treatment of self-limiting conditions (Thormar, 2011). *Eucalyptus* essential oil is commonly sold in pharmacies and retail outlets in the form of ointments, sprays, and lozenges. The oil may also be found in combination with other essential oils, incorporated into cleaning products for its disinfectant properties, sold as a chest rub for respiratory conditions **(**Jones *et al.*, 2007; Heinrich and Jäger, 2015). Some of the well-known commercial products containing *Eucalyptus* oil or its components (for example, 1,8-cineole, linalool, limonene) include: Ingram's Camphor cream Herbal® (Tiger Brands) or Lux® hand and body lotion (Unilever)

both containing linalool and limonene, Airwaves Menthol *Eucalyptus*® sugar free chewing gum, Colgate Herbal White® toothpaste, Strepsils Menthol and *Eucalytpus*®, Cool Mint® Listerine® Antiseptic Mouthwash (eucalyptol), Vicks® VapoRub®, Close Up Deep Action *Eucalyptus* Mint Toothpaste®.

The *Eucalyptus* genus (Myrtaceae family) has been cultivated for many years (Luqman *et al.*, 2008; Ali *et al.*, 2015). The *Eucalyptus* essential oil industry was initiated 163 years ago, in 1852 by a pharmacist named Joseph Bosisto (Pearson, 1993; Guba, 2009). Bosisto established a distillation plant which he used to manufacture a range of medicinal products. The first *Eucalyptus* essential oil to be produced was *E. radiata* essential oil (Pearson, 1993; Guba, 2009). *Eucalyptus radiata* leaf essential oil is therefore, one of the oldest sources of commercial *Eucalyptus* essential oils (Doran *et al.*, 1998). Today, *Eucalyptus* essential oil is in the top 20 of the most commonly used and produced essential oils in the world (Lawrence, 1993; Trade and Industrial Policy Strategies and Australian Agency for International Development, 2008).

The *Eucalyptus* genus comprises over 600 species (Jones *et al.*, 2007). Of the > 600 species, approximately 20 are used for commercial oil production (Pearson, 1993; Jones *et al.*, 2007). The commercial use of the oil is determined by the chemical composition, which in turn varies with each *Eucalyptus* species (Jones *et al.*, 2007). There are three types of *Eucalyptus* essential oils: medicinal, industrial and perfumery/flavouring oils (Pearson, 1993; Subramanian *et al.*, 2012). Medicinal oils are characterised by a high 1,8-cineole content. Industrial oils are characterised by a high piperitone and phellandrene content and perfumery/flavouring oils are characterised by a high citronellal and geranyl acetate content (Pearson, 1993).

The most important oil is the 1,8-cineole rich oil, also known as the medicinal type oil (Jones *et al.*, 2007). For this study, further discussion will focus on the medicinal type *Eucalyptus* essential oil. According to the British Pharmacopoeia, a minimum cineole content of 70% is required to classify the oil as medicinal type oil (Chisowa, 1997; British Pharmacopoiea, 2015). Other marker compounds for the medicinal type oil are: α-pinene; β-pinene and limonene (British Pharmacopoiea, 2015). The species mainly used as sources of medicinal

Eucalyptus essential oils are; *Eucalyptus globulus* Labill., *Eucalyptus polybractea* R.T.Baker and *Eucalyptus smithii* R.T.Baker and *E. camaldulensis* (Jones *et al.*, 2007; British Pharmacopoiea, 2015). *Eucalyptus globulus* is the most popular source of medicinal *Eucalyptus* essential oil (Bajaj, 1995; Shankaranarayana *et al.*, 2006; Jones *et al.*, 2007) and is used in the British Pharmacopoeia as the standard chemical profile (chemotype) for medicinal *Eucalyptus* oil (Bajaj, 1995).

1.4.2. Yield and chemical composition of *Eucalyptus* essential oils

There is a large variation in essential oil yield and composition between species of the *Eucalyptus* genus, as a result of genetic and non-genetic factors (Coppen and Hone, 1992). An example of a genetic difference would be the existence of chemotypes (Table 1.1). Due to genetic factors, some species naturally produce oil yields and 1,8-cineole in higher quantities in comparison to others (Table 1.2). However, other than genetics, previous studies have shown how non-genetic factors such as leaf age (harvest time) and seasonal variation can affect the quality (yield) and quantity (chemical composition) of the oils of some *Eucalyptus* species (Li *et al.*, 1996; da Cruz Francisco *et al.*, 2001; Silva *et al.*, 2003; Sartorelli *et al.*, 2007; Sefidkon *et al.*, 2010; Jemaa *et al.*, 2012).

Table 1.2. *Eucalyptus* species utilised for commercial oil production as cited by Pearson, 2003.

Species	Essential oil yield (%)	Major compound (%)	Hits on Sciencedirect (2015 search)
Medicinal oil			
E. globulus	0.7 - 2.4	1,8-Cineole 60 - 85	2718
E. radiata	2.5 - 3.5	1,8-Cineole 65 - 75	108*
E. dives	3.0 - 6.0	1,8-Cineole 60 - 75	84**
E. sideroxylon	0.5 - 2.5	1,8-Cineole 60 - 75	64
E. polybractea	0.7 - 5.0	1,8-Cineole 60 - 93	60
E. smithii	1.0 - 2.2	1,8-Cineole 70 - 80	36
E. leucoxylon	0.8 - 2.5	1,8-Cineole 65 - 75	33
E. amygdalina	***	1,8-Cineole and pinene and phellandrene	33
E. cinerea	± 1.2	1,8-Cineole	29
E. consideniana	-	Cineole and phellandrene	10
E. elaeophora	1.5 - 2.5	1,8-Cineole 60 - 80	8
E. viridis	1.0 - 1.5	1,8-Cineole 70 - 80	7
E. cnerifolia	2.0	1,8-Cineole 40 - 90	6
E. morrisii	± 1.6	1,8-Cineole	***
Industrial oils			

Species	Essential oil yield (%)	Major compound (%)	Hits on Sciencedirect (2015 search)
E. dives	1.5 - 5.0	Phellandrene 60 -80	**
	3.0 - 6.5	Piperitone 40 - 56	**
E. radiata	± 3.5	Phellandrene 35 - 40	*
E. elata	± 3.5	Piperitone 40 - 50	15
Perfumery and flavoring oils			
E. citriodora	0.5 - 2.0	Citronellal 65 - 80	339
E. macarthurii	0.2 - 1.0	Geranyl acetate 60 -70	15

* and ** = Same values indicated for the same species; ± = Approxiamately; *** = No values stated in literature; - = no value..

1.4.2.1. Influence of seasonal variation on the yield and chemical composition of *Eucalyptus* essential oil

Moudachirou *et al.* (1999) reported high essential oil yields in February/March and April/May for *E. camaldulensis* samples collected from Calavi and Ketou respectively. In comparison, Calavi *E. citriodora* samples yielded much higher essential oil than Ketou samples regardless of seasonal variation (Moudachirou *et al.*, 1999). This showed that, for *E. camaldulensis*, the yield is dependent on both seasonal variation (harvest) and location, whereas for *E. citriodora*, yields are mostly dependent on location. Silva *et al.* (2006) identified that the relationship between yield and seasonal variation was linked to the changes in water content (rainfall) and temperature between seasons. For example, nine out of the eleven species assessed presented low yields in spring (water deficient season) while higher yields were obtained in summer (characterized by high rainfall and high temperatures) (Silva *et al.*, 2006).

In contrast, Sefidkon *et al.* (2009) noted that the best harvesting time for a 1,8-cineole rich oil was spring, autumn and winter for *E. porosa*, *E. leucoxylon* and *E. camaldulensis* respectively. In contrast, seasonal variation had no significant effect on the 1,8-cineole content of the essential oils of *E. kochii* and *E. plenissima* (Brooker *et al.*, 1988). The effects of seasonal variation on chemical composition are mainly quantitative rather than qualitative for some *Eucalyptus* oils. For example, the major compounds of 10 *Eucalyptus* species sampled in autumn, winter, spring and summer varied in ratio but remained the same compounds regardless of seasonal variation (Silva *et al.*, 2006). These observations in literature show that the chemical composition of the oils of different *Eucalyptus* species is affected differently by seasonal variation. In addition, under different growth conditions

(seasonal variation) some *Eucalyptus* species may yield a different quantity and quality of essential oil.

1.4.2.2. Influence of leaf age on the yield and chemical composition of *Eucalyptus* essential oil

Li *et al.* (1996) noted that the differences in young and mature leaf oil composition were mainly quantitative rather than qualitative upon evaluation of 17 *Eucalyptus* species. In contrast, Sartorelli *et al.*, 2007 identified both quantitative and qualitative differences between young and mature *E. saligna* leaf oil samples. Young *E. saligna* leaf oil contained *p*-cymene (54.20%) and γ-terpinene (43.80%) as the major compounds, while mature *E. saligna* leaf oil contained α-pinene (45.10%) and lower amounts of *p*-cymene decreased (22.50%) as major compounds (Sartorelli *et al.*, 2007). α-Pinene (45.1%) was present in the young *E. saligna* sample but absent in the mature *E. saligna* leaf oil sample (Sartorelli *et al.*, 2007). These observations show that, for *Eucalyptus* oils variation in chemical composition due to leaf age/maturity may occur. However, the significance of leaf age on the chemical composition of the essential oil varies with species.

1.4.3. Antimicrobial activity of *Eucalyptus* essential oil

Eucalyptus essential oils and their compounds (e.g. 1,8-cineole, pinene, limonene, piperitone and globulol) have demonstrated antimicrobial activity, against a broad-spectrum of micro-organisms (Cermelli *et al.*, 2007; van Vuuren and Viljoen, 2007; Batish *et al.*, 2008; Agarwal and Lakshmi, 2013; Kerekes *et al.*, 2013; Chaudhari *et al.*, 2014; Ali *et al.*, 2015). These included pathogens associated with respiratory conditions such as *Streptococcus pyogenes, Streptococcus agalactiae, S. pneumoniae, Cryptococcus neoformans, K. pneumoniae, M. catarrhalis* (Inouye *et al.*, 2001; Cermelli *et al.*, 2007; Luqman *et al.*, 2008; Agarwal and Lakshmi, 2013). Pathogens associated with dental pathogens such as *Porphyromonas gingivalis, Streptococcus sobrinus, Streptococcus mutans* (Takarada *et al.*, 2004; Chaudhari *et al.*, 2014; Golestannejad *et al.*, 2015). Pathogens associated with wound infections such as *C. albicans, S. aureus, P. aeruginosa* and gastrointestinal/food related pathogens such as; *Bacillus cereus, E. coli, Salmonella typhimurium, Listeria monocytogenes* (Lis-Balchin and

Deans, 1997; Damjanovic-Vratnica *et al.*, 2011; Kumar Tyagi *et al.*, 2014; Sharafati Chaleshtori *et al.*, 2015; Sebei *et al.*, 2015).

Previous studies have shown that the antimicrobial activity of *Eucalyptus* oils varies with different microbial species (Elaissi *et al.*, 2011; Kumar Tyagi *et al.*, 2014). Luqman *et al.* (2008) tested the antimicrobial activity of *E. citriodora* and found that the oil exhibited higher activity against fungi (MIC ranged 1.25 - 10.00 mg/ml) in comparison to bacteria (MIC ranged ≥ 10 mg/ml).

Antimicrobial activity of *Eucalyptus* oils also varies within microbial strains (Elaissi *et al.*, 2011; Kumar Tyagi *et al.*, 2014). According to Lis-Balchin and Deans 1997), *E. radiata, E. globulus* and *E. citriodora* essential oils showed inhibitory activity against 20, six, and 20 *L. monocytogenes* strains, respectively. What is also interesting to note is that, although *E. radiata* (84.0% 1,8-cineole) and *E. citriodora* (0.005% 1,8-cineole) had different amounts of 1,8-cineole, they inhibited all 20 *Listeria* strains. In contrast *E. globulus* (90.8% 1,8-cineole) had the lowest anti-*Listeria* action, although it had a similar 1,8-cineole content to *E. radiata* in comparison to *E. citriodora* (Lis-Balchin and Deans, 1997). This observation is in contrast with the observation that antimicrobial activity is associated with the major compounds (e.g 1,8-cineole). Instead, this is an indication that antimicrobial activity may be associated with the result of interactions between major and minor compounds within the essential oils (Kumar Tyagi *et al.*, 2014). A comparison of the antimicrobial activity of *E. globulus* with its major compound, 1,8-cineole showed that, 1,8-cineole displayed a greater than or equal antimicrobial activity to the *E. globulus* essential oil depending on the test pathogens. This finding also confirmed that even though the major compound 1,8-cineole may play a significant role in the antimicrobial activity, minor compounds within the essential oil also contribute to the overall activity (Hendry *et al.*, 2009). Van Vuuren and Viljoen (2007) evaluated the antimicrobial activity of 1,8-cineole and limonene independently and in combination. Their study confirmed the presence of interactions between compounds and further identified the significance of compound ratios on the outcome of the interactions.

Medicinal *Eucalyptus* oil has great anti-infectious potential. This warrants the investigation of these lesser-known species concerning their chemical composition, antimicrobial activity and

antiquorum sensing activity as potential sources of additional/alternative medicinal *Eucalyptus* essential oil.

1.5. *Eucalyptus radiata* essential oil

1.5.1. Nomenclature and description

Eucalyptus radiata (syn. *Eucalyptus australiana*) is commonly known as the 'narrow-leaved peppermint'. Other common names include; 'forth river peppermint', 'grey peppermint' or 'black peppermint' (Doran *et al.*, 1998; Stewart, 2005; Rankin, 2009; Williams, 2011; Tourles, 2012). This species grows as an evergreen tree, as with nearly all *Eucalyptus* species (Kumar and Laxmidhar, 2011). Young and mature leaves of this plant are phenotypically distinct. Leaves of the young leaf are sessile, cordate-ovate and grow on opposite sides of the branch (Figure 1.2a). In contrast, the mature leaves are petiolate, lanceolate and grow on alternate sides of the branch, generally presenting with a darker shade of green in comparison with the young leaf (Figure 1.2b).

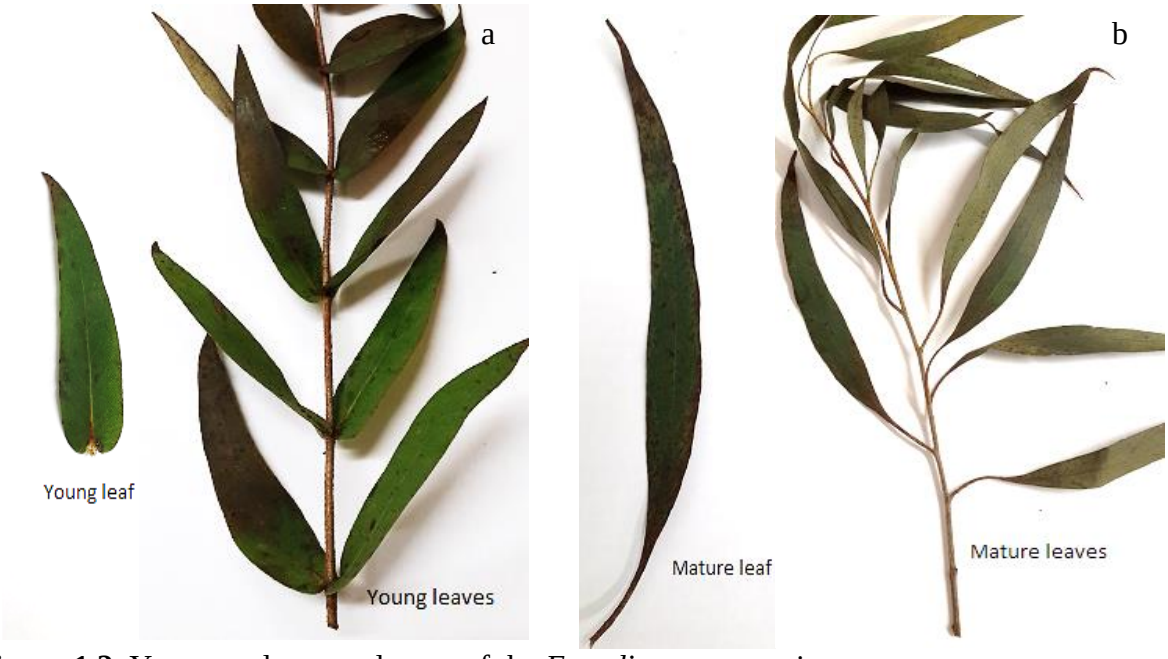

Figure 1.2. Young and mature leaves of the *E. radiata* tree species.

1.5.2. Uses and therapeutic relevance of *E. radiata* essential oil

Eucalyptus radiata essential oil is a highly valuable essential oil in aromatherapy. *Eucalyptus radiata* is one of the main *Eucalyptus* species used in aromatherapy (Balz, 1999; Farrer-Halls, 2009). *Eucalyptus radiata* is used interchangeably with the popular *E. globulus,* and is often preferred by aroma therapists due to its pleasant fragrance and cooling property in comparison to the popular *E. globulus* species (Mulyaningsih *et al.*, 2011). Its fragrant aroma has been attributed to the presence of citral in the essential oil composition (Doran *et al.*, 1998; Guba, 2009).

Various pharmacological and medicinal properties have been attributed to the *E. radiata* leaf essential oil. These include anti-inflammatory, antimicrobial, antiviral, mucolytic, immune stimulant, anticatarrhal, antiseptic, antitussive, neurotonic, as an energiser, expectorant and decongestant (Balz, 1999; Zhiri and Baudoux, 2005; Kovac, 2011). In addition, the *E. radiata* species is traditionally used as a remedy for; skin conditions (e.g. acne, wound healing and wound infections, abscess), respiratory conditions (bronchitis, colds, influenza, nasal congestion, asthma-like bronchitis, sinusitis, sinus infections), genitourinary conditions (leucorrhoea, prostatitis, cystitis, kidney infection, vaginitis), dental conditions (gingivitis), upper abdominal pain, otitis, rheumatism, strains, amongst other conditions (Balz, 1999; Zhiri and Baudoux, 2005; Kovac, 2011; Mulyanignsih *et al.*, 2011; Synovitz and Larson, 2012). The most extensive use of *E. radiata* leaf essential oil is for respiratory conditions (Rose and Earle, 1996; Stewart, 2005; Mulyaningsih *et al.*, 2011). In aromatherapy, *Eucalyptus radiata* essential oil is used independently or in combination with other essential oils for the treatment of a variety of conditions (Rose and Earle, 1996; Higley and Higley, 1998; Balz *et al.*, 1999; Kovac, 2011; Synovitz and Larson, 2012***).*** Table 1.3 shows some essential oils used in combination with *E. radiata* leaf essential oil in aromatherapy and their indications. According to Guba (2009), *E. radiata* oil can be used in 'synergy' with *Eugenia caryophyllus* (clove), *M. alternifolia* (tea tree), *A. fragrans* (fragonia), *C. martini* (palmarosa), *O. majorana* (sweet marjoram), *T. vulgaris* (sweet thyme) and *T. vulgaris* (red thyme) for their anti-bacterial properties. However, there have been no studies presented to confirm any synergy or the effects of *E. radiata* essential oil on the efficacy of other oils. Due to its frequency of use

in combination blends, it is necessary to evaluate whether it has the ability to enhance the antimicrobial/therapeutic properties of other essentials in antiinfective blends.

In addition to its ethnomedicinal relevance, *E. radiata* is a significant commercial source of 1,8-cineole rich oil. Globally, *E. radiata* is one of the 20 commercially relevant sources of medicinal essential oil, Table 1.2 (Pearson, 1993; Pech, 2006; Stewart, 2005; Guba, 2009). The majority of medicinal type oil (high 1,8-cineole content) production falls outside Australia (the native region of *Eucalyptus* plants) in countries like South Africa. In South Africa, *E. radiata* is one of the two main sources of medicinal *Eucalyptus* essential oil (Coppen and Hone, 1992).

The essential oil yields are estimated to be higher than the popular *E. globulus*, and the current source of medicinal type oil in South Africa; *E. smithii*, Table 1.2. In spite of this, *E. radiata* essential oil constitutes a small contribution in the commercial medicinal *Eucalyptus* oil market in South Africa. Furthermore, despite its medicinal and commercial relevance, very little is known with regards to the yield, chemical composition and antimicrobial properties of South African harvested *E. radiata* essential oil.

1.5.3. Yield and chemical composition of *E. radiata* leaf essential oil

1.5.3.1. Factors influencing *E. radiata* essential oil yield

Outside of South Africa, *Eucalyptus radiata* leaf essential oil has been studied with regards to yield and chemical composition. *Eucalyptus radiata* is one of the high essential oil yielding *Eucalyptus* species (Coppen and Hone, 1992; Pearson, 1993; Bajaj, 1995; Doran *et al.*, 1998). The expected essential oil yields are estimated to be in the range between 2.50% - 3.50% (Doran *et al.*, 1998; Pearson, 1993). However, yields as high as 9.00% have been previously reported (Chisowa, 1997). This variation in yield has been attributed to the ability of the species to increase yields under optimal conditions (Doran *et al.*, 1998). This is an indication of the role of non-genetic factors such as growth conditions and seasonal variation on the resultant yield of this species. Singh (1994), noted a 6.70% - 8.40% variation

Table 1.3. Essential oils used in combination with *E. radiata* essential oil for infectious conditions.

Indication	Administration	Combination	Reference
Sinus infection	Inhalation Topical	*Eucalyptus radiata* *Mentha piperita*	Rose and Earle, 1996
Respiratory blend	Topical (Massage into chest area)	*Eucalyptus radiata: Lavandula lactifolia, Rosmarinus officinalis*	Martin, 2007
Sinus congestion	Inhalation (inhaler stick)	*Eucalyptus radiata: Mentha piperita* *Rosmarinus officinalis*	
Influenza	Topical (chest rub)	*Eucalyptus radiata: Cinnamomum camphora, Melaleuca quinquenervia*	Kindergoatsoaps.com, (no date)
Otitis	Topical (rub around ear)	*Eucalytpus radiata: Melaleuca alternifolia, Lavandula burnati*	
Herpes simplex (cold sores and genital herpes)	Topical	*Eucalyptus radiata: Citrus bergamia*	
Influenza and bronchitis	Inhalation	*Eucalyptus radiata*: *Thymus vulgaris*, *Abies concolor, Lavandula angustifolia*	
Sore throat	Topical (Massage oil)	*Eucalyptus radiata* *Lavandula angustifolia* *Perlagonium asperum, Thymus vulgaris,* Coconut oil	
Sinus inhalant: (keep sinuses clear and open)	Inhalation	*Eucalyptus radiata: Origanum vulgare, Citrus sinensis, Eugenia caryophyllus*	
Athletes foot	Topical	*Eucalyptus radiata: Chamaemelum nobile, Commiphora molmol, Melaleuca alternifolia*	
Ear infection	Topical (massage oil)	*Eucalyptus radiata: Melaleuca alternifolia*	Fragranceofessences.com, 2015
Sore throat	Topical	*Eucalyptus radiata:* Ravintsara, Scots pine essential	
Bronchitis	Topical	*Eucalyptus radiata: Ravintsara, Scots pine essential*	
Respiratory (viral infections)	Topical	*Eucalyptus radiata: Cinnamomum camphora*	Rhind, 2012); Schnaubelt, 2011
Sinus infection	Inhalation Topical	*Eucalyptus radiata: Mentha piperita*	Rose and Earle (1996)
Sore throat	Inhalation Topical	*Eucalyptus radiata: Mentha piperita, Melaleuca alternifolia*	
Cold sores and herpes	Topical	*Eucalyptus radiata: Citrus bergamia*	Farrer-Halls, 2009
*	*	*Eucalyptus radiata*: *Eugenia caryophyllus*, *Melaleuca alternifolia, Agonis fragrans, Cymbopogon martini, Origanum majorana, Thymus vulgaris,* (linalool), *Thymus vulgaris,* (thymol)	Guba, 2009

ND = not dated; * = Data not stated in literature.

in essential oil yield in an *E. radiata* species, confirming the influence of seasonal variation on chemical composition. Other than Singh (1994), there has been no other study evaluating

the effect of seasonal variation on *E. radiata* oil yield to date. Due to the lack of seasonal variation studies on the essential oil yield, very little is known about what these optimal conditions are.

Both young and mature leaf glands contain essential oils (Doran *et al.,* 2005). This means that both young and mature leaves of the *E. radiata* species can be used as essential oil sources. For commercial essential oil producers, this translates to higher yield potential (oil from young and mature leaves versus young or old leaves only) and low waste. Analysis of the effects of seasonal variation and leaf age of *E. radiata* oil is required in order to provide insight into what these ideal growth conditions might be, should commercial interest arise.

1.5.3.2. Factors influencing the chemical composition of *Eucalyptus radiata* leaf essential oil

Intra-specific variation in essential oil composition abounds with *E. radiata* species oil (Doran *et al.*, 1998; Guba, 2009; Doran *et al.,* 2005). At least six chemotypes have been reported for *E. radiata* , Table 1.1 (Doran *et al.,* 1998; Coppen, 2002; Stewart, 2005; Guba, 2009). However, only two of these chemotypes are of commercial interest: the piperitone/phellandrene-rich chemotype used for industrial purpose and the 1,8-cineole-rich chemotype used for medicinal purpose. This highlights the commercial relevance of *E. radiata* oil with its broad potential for application in the pharmaceutical industry ranging from medicinal products to disinfectants.

Eucalyptus radiata essential oil is a rich source of phytochemicals (Table 1.4). These earlier reports identified a range of 23 to 35 compounds. The essential oil is a hetero-chemical cocktail of: monoterpenes (for example, α-pinene, β-pinene, limonene, myrcene, sabinene, γ-terpinene), oxides (1,8-cineole) sesquiterpenes (for example, β-caryophyllene), monoterpene alcohols (for example, linalool, α-terpineol, terpinene-4-ol, nerol, geraniol), aldehydes (geranial, neral).

1,8-Cineole, and α-terpineol have been identified as the main components of medicinal *E. radiata* essential oil (Balz, 1999; Stewart, 2005; Zhiri and Baudoux, 2005; Guba, 2009). This

is in agreement with the summary of the chemical profile of medicinal *E. radiata* essential oil samples from various parts of the world (Table 1.4). There are some key compounds identified in the chemical profile of *E. radiata* oil such as: 1,8-cineole, α-terpineol, α-pinene and limonene. These compounds were present in high ratios relative to other compounds and were present in the majority of essential oil samples from previous studies (Table 1.4). However, no two *E. radiata* oil samples had the exact same chemical composition, even though all essential oils were from the same species (Table 1.4). *Eucalyptus radiata* samples from different regions across the world showed a variation in the ratio of minor and major compounds. This confirms that the composition of the *E. radiata* oil may vary, as observed with other *Eucalyptus* species. The samples noted in Table 1.4 represent sample from different regions across the world (for example, Germany, Tunisia, Zambia, Portugal and India). These *E. radiata* plant sources were therefore exposed to varied growth conditions and seasonal variations amongst other things. Therefore, the variation in chemical composition observed may be as a result of these differences in growth conditions. There has been no study evaluating the effect of the unique South African climate on *E. radiata* oil composition to date.

1.5.4. Antimicrobial activity of *Eucalyptus radiata* leaf essential oil

Eucalyptus radiata oil has shown to exhibit a broad-spectrum of activity (Table 1.5). This includes aerobic and anaerobic micro-organisms, Gram-positive and Gram-negative bacterial strains, as well as and fungal strains. However, some of these studies were conducted using out-dated screening methods such as the diffusion assay. Previous antimicrobial studies on *E. radiata* included measures of vapour activity (Inouye *et al.*, 2001), diffusion assays (Lis-Balchin and Deans, 1997; Bendaoud *et al.*, 2009) and the quantitative microdilution minimum inhibitory concentration (MIC) assay (Takarada *et al.*, 2004; Mulyaningsih *et al.*, 2011; Luís *et al.*, 2015). A number of limitations arise from some of these methods of analysis. *Eucalyptus radiata* essential oil comprises volatile compounds. The difference in volatility of various compounds within the essential oil may not only affect the composition of the vapour (Laird and Phillips, 2012) but will also lead to loss of a portion of the essential oil during the pre-diffusion stage in an agar diffusion assay (van Vuuren, 2008).

Table 1.4. Percentage composition (%) of *Eucalyptus radiata* essential oil studies.

Compound	References and chemical composition of *E. radiata* oil samples							
	Singh, 1994 (India)	**Chisowa, 1997 (Zambia)**	**da Cruz Francisco *et al.*, 2001 (Australia)**	**Bendaoud *et al.*, 2009 (Tunisia)**	**Guba, 2009 (Australia)**	**Juan *et al.*, 2011 (Auatralia)**	**Mulyaningsih *et al.*, 2011 (Germany)**	**Luís *et al.*, 2015 (Portugal)**
Terpenes and terpenoids								
(E)-β-Ocimene	*	0.20	*	*	*	*	*	*
Camphene	*	*	*	0.06	*	*	*	*
Limonene	**4.50**	**3.70**	**5.59**	0.45	**3.75**	**7.32**	*	**68.51**
Myrcene	*	1.00	*	*	1.32	*	*	*
O-Cymene	*	*	*	*	*	*	*	0.69
p-Cymene	*	0.10	*	1.96	*	0.70	*	0.51
Pinocarvone	*	*	*	1.31	*	*	*	*
Sabinene	*	1.10	*	0.86	1.06	*	1.40	0.97
Terpinolene	*	tr	*	*	*	*	tr	*
α-Phellandrene	*	*	*	*	*	0.19	*	*
α-Pinene	*	1.70	2.59	**11.94**	2.25	2.80	3.68	3.01
α-Terpinene	*	0.20	1.39	*	*	*	*	
α-Thujene	*	*	*	*	*	*	*	0.18
β-Myrcene	*	*	*	0.32	*	*	*	*
β-Ocimene	*	*	*	*	*			0.07
β-Pinene	*	0.50	*	*	0.64	*	*	1.13
Hexenyl acetate	*	tr	*	*	*	*	*	*
γ-Terpinene	*	0.30	*	0.25	*	0.53	tr	*

Compound	References and chemical composition of *E. radiata* oil samples							
	Singh, 1994 (India)	**Chisowa, 1997 (Zambia)**	**da Cruz Francisco *et al.*, 2001 (Australia)**	**Bendaoud *et al.*, 2009 (Tunisia)**	**Guba, 2009 (Australia)**	**Juan *et al.*, 2011 (Auatralia)**	**Mulyaningsih *et al.*, 2011 (Germany)**	**Luís *et al.*, 2015 (Portugal)**
α-Terpinyl acetate	*	1.00	*	*	2.37	*	*	6.07
Isoamyl isovalerate	*	*	*	0.10	*	*	*	*
Trans-*p*-Menth-2-en-1-ol	*	*	*	*	*	0.06	*	*
Endo-Fenchol	*	*	*	0.11	*	*	*	*
4-Terpineol	*	*	*	*	*	1.84	*	*
cis-*ρ*-Menth-2-en-1-ol	*	*	*	*	*	0.09	*	*
Geranial	*	0.20	*	*	1.04	*	*	0.61
Geraniol	*	0.80	*	*	0.24	*	*	*
α-Terpineol	**11.60**	**6.40**	**7.95**	*	**8.65**	**12.44**	**7.03**	**8.60**
Nerol	*	*	*	*	0.10	*	*	0.08
1.8-Cineole	**74.25**	**80.80**	**76.36**	**69.53**	**71.28**	**68.36**	**82.66**	*****
Camphor	*	*	*	0.20	*	*	*	*
Carvacrol	*	*	*	0.03	*	*	*	*
Borneol	*	*	*	0.21	*	*	*	*
trans-Pinocarveol	*	*	*****	**4.81**	*	*	*	*
Cryptone	*	*	*	*	*	*	*	0.13
p-Cymen-7-ol[a]	*	*	*	0.09	*	*	*	*
p-Cymen-8-ol[a]	*	*	*	0.35	*	*	*	*
Linalool	*	0.40	*	*	0.34	*	tr	0.40

Compound	References and chemical composition of *E. radiata* oil samples							
	Singh, 1994 (India)	**Chisowa, 1997 (Zambia)**	**da Cruz Francisco *et al.*, 2001 (Australia)**	**Bendaoud *et al.*, 2009 (Tunisia)**	**Guba, 2009 (Australia)**	**Juan *et al.*, 2011 (Auatralia)**	**Mulyaningsih *et al.*, 2011 (Germany)**	**Luís *et al.*, 2015 (Portugal)**
Myrtenol	*		*	0.07		*	*	*
Terpinene-4-ol	*	0.70	1.30	*	2.15	*	1.53	1.61
(3Z)-Hexenyl angelate	*	*	*	0.02	*	*	*	*
Exe-2-hydroxy cineole acetate	*	*	*	*	*	*	*	0.10
γ-Terpineol	*	*	*	*	0.54	*	*	*
Bicyclogermacrene	*	*	*	*	*	*	*	
δ-Terpineol	*	*	1.31	*	0.18	*	*	0.25
cis-Limonene oxide	*	*	*	*	*	*	*	*
Verbenone	*		*	0.35		*	*	*
Methyl-E-cinnamate	*	*	*	*	*	*	*	0.19
p -Menth-3,8-diene	*	*	*	*	*	*	*	*
Pulegone	*	*	*	0.11	*	*	*	*
Neral	*	0.20	*	*	0.82	*	*	0.52
α -Selinene	*	*	*	0.03		*	*	*
Gurjunene	*	*	*	0.02	*	*	*	*
α -Humulene	*	*	*	*	*	*	*	*
γ -Muurolene	*	*	*	0.04		*	*	*
β-Selinene	*	*	*	2.62		*	*	*
δ-Cadinene	*	*	*	*	*	*	0.55	*
α-Caryophyllene	*	*	*	*	*	*	0.04	*

Compound	References and chemical composition of *E. radiata* oil samples							
	Singh, 1994 (India)	**Chisowa, 1997 (Zambia)**	**da Cruz Francisco *et al.*, 2001 (Australia)**	**Bendaoud *et al.*, 2009 (Tunisia)**	**Guba, 2009 (Australia)**	**Juan *et al.*, 2011 (Auatralia)**	**Mulyaningsih *et al.*, 2011 (Germany)**	**Luís *et al.*, 2015 (Portugal)**
β-Bourbonene	*	*	*	0.29	*	*	*	*
β-Caryophyllene	*	*	*	0.02	*	*	*	0.25
Viridiflorene	*	*	*	*	*	*	*	*
Aromadendrene	*	*	*	*	*	*	*	0.14
α-Cubebene	*	*	*	0.26	*	*	*	*
Ledol	*	*	*	*	*	*	*	0.05
α -Himachalene	*	*	*	0.06	*	*	*	*
Eremophyllene	*	*	*	*	*	*	*	0.10
β-Caryophyllene oxide	*	*	*	0.06	*	*	*	*
γ -Eudesmol	*	tr	*	*	*	*	*	*
β-Eudesmol	*	tr	*	0.44	*	*	*	*
Viridiflorol	*	*	*	0.11	*	0.23	*	0.10
Caryophyllene oxide	*	*	*	*	*	*	*	0.10
Elemol	*	*	*	0.07	*	*	*	*
Spathulenol	*	*	*	*	*	*	tr	
(-)-Spathulenol	*	*	*	*	*	*	*	0.24
Globulol	*	*	*	*	*	*	*	0.15
α -Eudesmol	*	tr	*	*	*	*	*	*
α-Cadinol	*	*	*	0.09	*	*	*	*
Other compounds								

Compound	References and chemical composition of *E. radiata* oil samples							
	Singh, 1994 (India)	**Chisowa, 1997 (Zambia)**	**da Cruz Francisco *et al.*, 2001 (Australia)**	**Bendaoud *et al.*, 2009 (Tunisia)**	**Guba, 2009 (Australia)**	**Juan *et al.*, 2011 (Auatralia)**	**Mulyaningsih *et al.*, 2011 (Germany)**	**Luís *et al.*, 2015 (Portugal)**
Other compounds	*	*	*	*	*	5.44	*	*
Equilenin (Steroid)	*	*	*	0.10	*	*	*	
Total area percentage (%)	90.35	99.30	96.49	97.34	96.73	100	96.89	94.76

tr = [t]trace amounts (< 0.10%); * = Value not stated in literature; bold = Major compound.

Furthermore, the physical-chemical properties of the compounds within the oil vary. This means that the end results would vary, based on the solubility of the compounds in the selected test media. Therefore, results of the vapour-phase method and agar diffusion assay would not be a true representation of the activity of the essential oil in relation to its chemical composition. Moreover, in many of the past studies, the micro-organisms screened did not account for the range of antiinfective therapeutic uses of the essential oil, therefore very little is still known about the rationale behind the essential oil's antiinfective properties.

Another consideration is that the lipophilic nature of essential oils may also affect diffusion through the agar medium (van Vuuren, 2008). Limitations of the vapour-activity and agar diffusion methods make these earlier results superflous. Therefore, only studies utilising the MIC method will be used for comparison of results in this study.

The antibacterial and antifungal activity of *E. radiata* was evaluated against two plant pathogenic bacteria (*Agrobacterium tumefaciens* and *Pseudomonas savastanoi*) and two plant pathogenic fungi *Fusarium solani* and *Rhizoctonia. solani*. Antimicrobial activity was observed against all micro-organisms, however, noteworthy antibacterial activity was observed against *A. tumefaciens* and *R. solani* with minimum inhibitory concentrations ranging between 750 - 1000 µl/l. For essential oils, noteworthy activity is regarded at MIC ≥ 2.00 mg/ml (van Vuuren, 2008). These results demonstrated the potential use of *E. radiata* oil as an alternative plant preservative agent (Bendaoud *et al.*, 2009).

According to Mulyaningsih *et al.* (2011), *E. radiata* essential oil is a potential alternative antimicrobial source against the multidrug-resistant *A. baumannii.* The essential oil demonstrated moderate activity against methicillin-resistant *S. aureus* (MIC value ≥ 4 mg/ml) and noteworthy antimicrobial activity against *A. baumannii* (MIC value ≥ 1 mg/ml), Table 1.5. *Eucalyptus radiata* essential oil showed antimicrobial activity against 20 *L. monocytogenes* strains, while the popular *E. globulus* showed activity against only six strains, this shows the potential role *E. radiata* could play in the food industry (Lis-Balchin and Deans, 1997). Takarada *et al.* (2004) noted the antimicrobial activity and anti-biofilm adhesion activity of *E. radiata* oil against the dental pathogen, *S. mutans*. This highlights the potential applications of the essential oil in dental care.

Table 1.5. Susceptibility data for micro-organisms tested against *E. radiata* leaf essential oil.

Micro-organism	Pathology	Dilution assay		Diffusion assay	Reference
		MIC	MBC	Zone of inhibition	
Listeria monocytogenes	Food spoilage	*	*	Diffusion assay used	Lis-Balchin and Deans, 1997
Haemophilus influenza	Respiratory tract pathogen	0.32 w/v	-	*	Inouye *et al.*, 2001
Porphytomonas gingivalis	Oral pathogen	0.25 - 0.50 v/v	0.25 - 0.50 v/v	*	Takarada *et al.*, 2004
Actinobacillus actinomycetemcomitans	Oral pathogen	0.50 v/v	0.50 v/v	*	
Fusobacterium nucleatum	Oral pathogen	0.13 - 0.25 v/v	0.50 v/v	*	
Streptococcus mutans	Oral pathogen	1.00 v/v	1.00 v/v	*	
Streptococcus sobrinus	Oral pathogen	1.00 v/v	1.00 v/v	*	
Methicillin-resistant *Staphylococcus aureus*	Nosocomial infections	4.00 mg/ml	*	26.00 mm	Mulyaningsih *et al.*, 2011
Agrobacterium tumefaciens	Plant pathogen	*	*	0.75 - 1.00 mg/ml	Bendaoud *et al.*, 2009
Pseudomonas savastanoi pv. Savastanol	Plant pathogen	*	*	0.75 - 1.00 mg/ml	
Fusarium solani	Plant pathogen	*	*	3.00 mg/ml	
Rhizoctonia solani	Plant pathogen	*	*	2.00 mg/ml	
Acinetobacter baumannii	Nosocomial infections	1.00 mg/ml	*	*	Mulyaningsih *et al.*, 2011
	*	8.00 mg/ml	8.00 mg/ml	*	Luís *et al.*, 2015
Pseudomonas aeruginosa	*	32.00 mg/ml	32.00 mg/ml	*	
Escherichia coli	*	16.00 mg/ml	*	*	
Klebsiella pneumoniae	*	16.00 mg/ml	*	*	
Salmonella typhimurium	*	32.00 mg/ml	*	*	

* = No results stated in literature.

1.5.4.1. Structure-activity relationship (SAR) of *E. radiata* leaf essential oil

The term ‘structure-activity relationship’ in this context refers to the correlation between chemical composition (‘structure’) and resultant antimicrobial activity. The chemical profiling of *E. radiata* oil in previous studies has revealed the presence of biologically active compounds such as 1,8-cineole, α-terpineol, linalool, geraniol, *p*-cymene and limonene amongst others. These compounds are known to have antimicrobial and antiquorum sensing

activity (Kotan *et al.*, 2007; van Vuuren and Viljoen, 2007; Mulyaningsih *et al.*, 2011; Bassole and Juliani, 2012; Djilani and Dicko, 2012; Andrade *et al.*, 2014; Ahmad *et al.*, 2014a and b).

For example, 1,8-cineole is well known for its expectorant properties, which correlates to the respiratory related and decongestant properties of *E. radiata* essential oil (Guba, 2009). As mentioned before, citral gives *E. radiata* its pleasant aroma. Linalool and α-terpineol showed strong antimicrobial activity against periondotopathic and cariogenic bacteria (Park *et al.*, 2012). Interestingly *E. radiata* oil showed noteworthy activity against dental pathogens. According to Kotan *et al.* (2007), nerol, linalool, α-terpineol, and terpinene-4-ol displayed activity against a number of microbial strains. The presence of these compounds within the oil might explain the wide range of antimicrobial activity observed in Table 1.5. Based on these observations, the essential oil exhibits therapeutic properties which are similar to the properties of its components. Therefore, there is a high probable correlation between the antimicrobial properties and potential antiquorum sensing activity of the essential oil and its chemical composition. However, there is very little scientific evidence evaluating the role of these compounds, and the effect of their interactions on the antimicrobial activity of the *E. radiata* leaf essential oil.

1.6. Study aims and objectives

The aim of this study was to evaluate the chemical composition and antimicrobial activity of young and mature leaf essential oils of *E. radiata* species. The antimicrobial activity of *E. radiata* leaf essential oil was compared to other commercially important *Eucalyptus* essential oils, should commercial interest arise. The antimicrobial and antiquorum sensing activity of *E. radiata* leaf essential oil was investigated in combination with other essential oils to determine potential synergistic activity. This study further examined the role of major compounds (independently and in combination) on the antimicrobial and antiquorum sensing activity of *E. radiata* leaf essential oil. The objectives of this study have been structured as follows;

- To collect young and mature *E. radiata* leaf samples every month for a period of one year (January 2014 to December 2014).

- Determine the chemical composition of *E. radiata* essential oil from both young and mature leaf samples collected monthly with the use of gas chromatography coupled with mass spectrometry (GC-MS).

- Determine the antimicrobial activity of the *E. radiata* essential oil samples from both young and mature leaf samples collected monthly from the period of January 2014 to December 2014 against a range of micro-organisms based on the therapeutic use of the essential oil using the minimum inhibitory concentration assay.

- Compare the antimicrobial efficacy of *E. radiata* leaf essential with leaf essential oils from other *Eucalyptus* species, namely; *E. globulus E. dives, E. smithii, E. citriodora* and *E. camaldulensis.*

- Determine the effect of *E. radiata* leaf essential oil on the antimicrobial activity of other essential oils at 1:1 combinations.

- Determine the antiquorum sensing activity of *E. radiata* leaf essential oil.

- Determine the effect of *E. radiata* leaf essential oil on the antiquorum sensing activity of other essential oils at 1:1 combinations.

- Determine the antimicrobial activity of major compounds independently and in combination using the fractional inhibitory concentration (ΣFIC).

- Examine the structure-activity relationship from the observed chemical composition and antimicrobial activity. A flow chart (Figure 1.3) representing how each objective was systematically followed is provided.

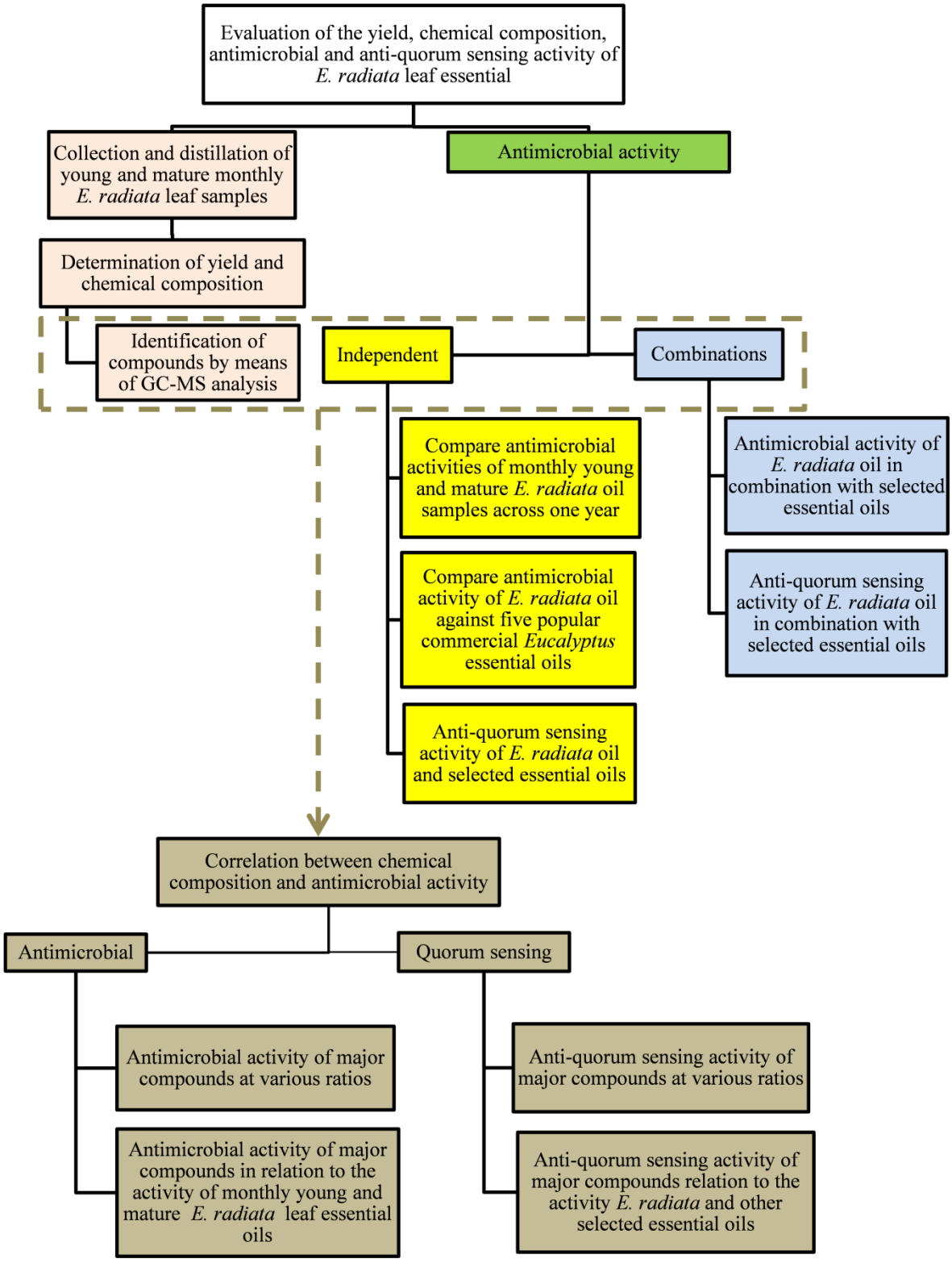

Figure 1.3. A schematic outline of the study for the evaluation of the antimicrobial activity and chemical composition of *E. radiata* leaf essential oil.

CHAPTER 2

Materials and methods

2.1. Plant collection and essential oil preparation

Fresh leaves of the *E. radiata* were collected from a cultivated site in Magoebaskloof, north of Polokwane, Limpopo Province, South Africa over a period of one calendar year (January 2014 to December 2014). In an effort to reduce the number of variables (i.e. geographical location, different growth conditions, differences in soil type) *E. radiata* leaves were collected within the same study area, from selected trees in the study site. Leaf material was identified and harvested under the supervision of the resident farmer, Mr. Bruce Stumbles.

During the sampling period, weather conditions in this region were characterised by high rainfall (35% average chance of precipitation) and high temperatures (average daily ± 27 ºC high and ± 17 ºC daily low) in summer and spring; and lower temperatures (average daily ± 19 ºC high and ± 7 ºC daily low) and low rainfall (4% average chance of precipitation) in autumn and winter (Historical weather for 2014 in Polokwane, South Africa [WWW Document], (2014). Seasonal variation, and varied growth conditions have been previously shown to affect the yield of *E. radiata* leaf essential oil, Table 1.4 (Singh, 1994). Therefore, monthly sampling was undertaken in order to assess how the unique weather conditions of the collection site affect the properties (yield and chemical composition) of the *E. radiata* species.

Previous studies have reported variation in essential oil composition between young and mature leaf oils of another *Eucalyptus* species, (*E. saligna*) (Sartorelli *et al.,* 2007). Therefore, the monthly plant samples comprised of both young and mature leaves to determine if variation due to leaf maturity exists within the *E. radiata* sample in this current study. Voucher specimens were collected for each monthly sample and recorded in the medicinal and aromatic plant register kept at the Department of Pharmacy and Pharmacology, University of the Witwatersrand.

2.1.1. Preparation of *E. radiata* leaf essential oil

Eucalyptus is traditionally prepared in the form of poultices or infusions, which can be consumed as teas or used via inhalation (Hylton *et al*., 1999). Poultices are traditionally used for topical application whereas infusions are typically used for respiratory ailments. The most widely known medicinal use of *Eucalyptus* essential oil is for respiratory ailments. The primary route of administration for respiratory treatment is via inhalation. Therefore, the most popular *Eucalyptus* preparation would be infusions. Infusions are prepared by immersing fresh leaves into boiling water; the resulting vapours are then inhaled or drunk as a tea. Both the tea and the vapours are believed to contain therapeutic properties. In addition, commercial *Eucalyptus* essential oil in South Africa is primarily prepared via the hydrodistillation method. Therefore, the *E. radiata* plant samples were distilled in order to mimic their traditional and commercial processing. This would not only allow simulation of the properties of the essential oil in the form of an infusion vapour, but would also allow for comparison of the antimicrobial activity of *E. radiata* leaf essential oil samples to commercial *Eucalyptus* oils. All *E. radiata* plant material was processed within 32 h of harvesting in order to prevent loss of any volatile compounds. The essential oil was obtained, as described by van Vuuren *et al.* (2014), using a Clevenger-type apparatus which was subject to hydrodistillation. Young and mature leaves were distilled separately throughout the sampling period. Figure, 2.1 is a graphical representation of the plant preparation process. Briefly, a known quantity (130 g - 1100 g) of weighed fresh leaf plant material was placed into 5000 L round bottom flasks (still). Approximately 900 L of distilled water was added into each still. Each still containing water and leaf material was then heated to boiling point (approximately 100 °C) using a heating mantle for a total of 3 h. Heating the water and plant mixture produces steam (water vapour) and oil. The water aids in carrying out the volatile oils and also aids in the prevention of overheating. The water vapour and volatile oil mixture was condensed to liquid form in the condensing tube (by the cool water running through it). After condensation, the water and essential oil mixture flowed from the condenser and collected in the receiving tube of the Clevenger apparatus. Essential oil is immiscible in water, resulting in a separation of the two liquids in the receiving tube. This allowed for easy separation of the water (hydrosol) and essential oil upon collection. This essential oil yield was quantified using Equation 2.1:

Equation 2.1

$$\text{Percentage yield} = \frac{\text{weight of essential oil}}{\text{weight of plant material}} \times 100$$

The determined essential oil yield was recorded in the medicinal and aromatic plant register kept at the Department of Pharmacy and Pharmacology, University of the Witwatersrand. Essential oils are volatile and need to be stored away from light in tightly sealed containers (Burt, 2004). Therefore, the essential oil samples were stored in tightly sealed amber bottles, away from light at ± 4°C until further analysis (Figure 2.1).

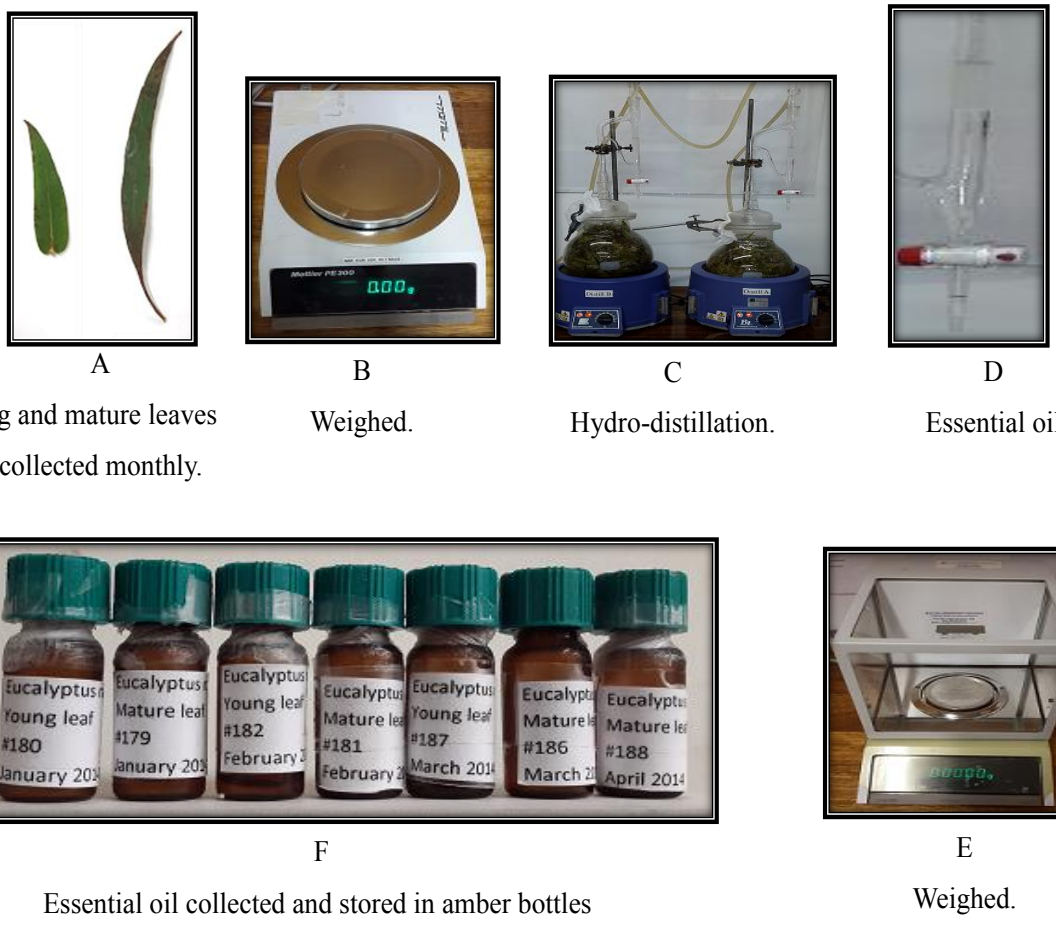

Figure 2.1. Plant preparation and hydrodistillation of young and mature *E. radiata* leaves using a Clevenger apparatus.

2.2. Commercial *Eucalyptus* essential oils

Selected essential oils from other *Eucalyptus* species were included in this study, as a means to evaluate the commercial potential, and efficacy of the *E. radiata* leaf essential oil by comparison to commercial *Eucalyptus* essential oils. *Eucalyptus camaldulensis* was also obtained using the hydrodistillation method. *E. camaldulensis* leaf material was collected from an area within the vicinity of Walter Sisulu botanical garden, Gauteng Province, South Africa. The *E. camaldulensis* leaf material was collected under the supervision of Andrew Hankey (curator), who confirmed the authenticity. *Eucalyptus globulus*, *E. dives*, *E. smithii*, *E. citriodora* and *Eucalyptus radiata* (herein referred to as *E. radiata*-comm) oil samples were all commercially acquired from Pranarôm (Belgium).

2.3. Essential oils used in combination with *E. radiata* essential oil

These oils were selected based on three criteria. First, there has to be literature evidence of the essential oils use in combination with *E. radiata* leaf essential oil for infectious conditions (Chapter 1, Table 1.5). This would allow for investigation of the interactive antimicrobial effects when the oils used in combination with *E. radiata* essential oil. Secondly, the essential oils had to have previously been screened for anti-quorum sensing activity. This would allow for investigation of the interactive anti-quorum sensing effects when the essential oils are combined with *E. radiata* essential oil. Lastly, the essential oils had to be available commercially. Accessibility contributes to the possibility of their concurrent use with *E. radiata* essential oil. The other essential oils selected for use with *E. radiata* leaf essential oil were commercially acquired from various sources (Table 2.1). Their composition was determined using gas chromatography coupled with mass spectrometry. Their major compounds are given in Table 2.1.

Table 2.1. The eleven essential oils obtained from commercial suppliers used in combination studies with *E. radiata*.

Essential oil	Source	Major compounds	Percentage composition
Citrus bergamia (bergamot)	Burgess and Finch	Limonene	39.00
		Linalyl acetate	33.00
		Linalool	9.20
Eugenia caryophyllus (clove)	Escentia products	Eugenol	81.90
		Eugenol acetate	13.13

Essential oil	Source	Major compounds	Percentage composition
		β-Caryophyllene	2.19
Lavandula angustifolia (lavender)	Burgess and Finch	Limonene	39.00
		Linalyl acetate	33.00
		Linalool	9.20
Citrus aurantifolia (lemon)	Escentia products	Limonene	74.90
		β-Pinene	7.20
		γ-pinene	5.50
Citrus limon (lime)	Burgess and Finch	α-Terpineol	16.40
		Terpinolene	11.20
		γ-terpinene	9.70
Leptospermum scoparium (manuka)	Natural solutions	Geranial	34.99
		Carveol	22.98
		Citronellol	15.42
Origanum marjorana (marjoram)	Burgess and Finch	1,8-Cineole	51.86
		Linalool	22.42
		α-Terpineol	4.50
Cymbopogan martini (palmarosa)	Burgess and Finch	Geraniol	80.70
		Linalool	2.97
Mentha piperita (peppermint)	Burgess and Finch	L-Menthol	47.03
		Menthone	18.75
		Menthyl acetate	5.95
Rosmarinus officinalis (rosemary)	Burgess and Finch	Verbenone	12.27
		Bornyl acetate	12.20
		1,8-Cineole	6.89
Melaleuca alternifolia (tea tree)	Escentia products	Terpinene-4-ol	35.00
		Terpinene	20.00
		α-Terpinene	10.00
Thymus vulgaris (thyme)	Burgess and Finch	*p*-Cymene	41.04
		Thymol	18.89
		γ-Terpinene	7.17

2.4. Chemical composition analysis

The essential oils were analysed by gas chromatography (Agilent 6890N GC) coupled directly to mass spectrometry and flame ionization detector (5973 MS) Figure 2.2. A volume of 1 µl was injected using a split ratio (200:1) with an auto-sampler at 24.79 psi and an inlet temperature of 250 °C. The GC system used was equipped with a HP-Innowax polyethylene glycol column 60 m × 250 µm i.d. × 0.25 µm film thickness. The oven temperature was set at 60 °C for the first 10 min, rising to 220 °C at a rate of 4 °C/min and held for 10 min; then rising to 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. Each peak area of the GC components represented the percentage composition of each individual component as a percentage of all the peak areas obtained from electronic integration measurements using flame ionization detection (FID, 250 °C). *n*-Alkanes were

used as reference points in the calculation of relative retention indices (RRI). A comparison of mass spectra from the: total ion chromatogram, retention indices and library searches using NIST® and Mass Finder® Flavor® libraries was used to identify the chemical components (van Vuuren *et al.*, 2014).

Figure 2.2. Gas chromatography coupled to mass spectrometry.

2.5. Antimicrobial assays

Two antimicrobial assays were undertaken. The first is the minimum inhibitory concentration (MIC) assay used to evaluate the antimicrobial activity. The second was the anti-quorum sensing assay used to screen for anti-quorum sensing activity. In both assays, essential oils and major compounds were evaluated independently, followed by investigation in combination. Independent screening of essential oils and major compounds was undertaken in order to obtain the necessary information to interpret the interactions observed in combination studies.

2.5.1. Preparation of media

Tryptone Soya broth (TSB) and Tryptone Soya agar (TSA) were used to culture bacterial and yeast strains with the exception of *Lactobacillus acidophilus* ATCC 314, *Chromobacterium violaceum* ATCC 12472 and the Streptococci. Mueller Hinton broth (MHB) was used to culture *L. acidophilus* and the Streptococci, exclusively. Luria Bertani broth (LBB) and Luria Bertani agar (LBA) were used to culture *C. violaceum* ATCC 12472 only. All media (TSB, TSA MHB, LBB and LBA (Oxoid)) was prepared in accordance with the manufacturer's instructions, i.e. weighed, dissolved in distilled water and autoclaved (Steridium) at 121 °C for 15 minutes. After sterilization, all media was left overnight at room temperature (25 °C ± 24 hours) in order to confirm sterility before further use. The sterility of the media was confirmed by the absence of turbidity in the media. Streptococci and *L. acidophilus*, do not grow well in MHB. Therefore, after confirmation of sterility, MHB was enriched with 5.00% sheep blood, to create a nutritious growth medium that would support the growth of these micro-organisms.

2.5.2. Preparation of culture

The *E. radiata* essential oil is used for a wide range of infectious conditions. These include its use as a remedy for acne, bronchitis, dental infections, wounds, respiratory problems, cystitis, sinus infections, vaginitis amongst other conditions (Higley and Higley, 1998; Balz *et al.*, 1999; Kovac, 2011). Therefore, the antimicrobial activity was evaluated against selected pathogens associated with the wide range of infectious conditions treated by the essential oil. These included the pathogens related to skin infections; (Gram-positive *Staphylococcus aureus* ATCC 25923, methicillin-resistant *Staphylococcus aureus* ATCC 33592, *Enterococcus faecalis* ATCC 29212; Gram-negative *Pseudomonas aeruginosa* ATCC 27853 and the yeast species *Candida albicans* ATCC 10231).

Gram-positive: *Bacillus cereus* ATCC 11778, *Listeria monocytogenes* ATCC 19111 and Gram-negative: *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028, and

Shigella sonnei ATCC 9290) were selected to represent pathogens related to gastrointestinal or food spoilage micro-organisms.

The Gram-positive: *Streptococcus pneumoniae* ATCC 49619, *Streptococcus agalactiae* ATCC 55618, *Streptococcus pyogenes* NHLS 8668, Gram-negative: *Klebsiella pneumoniae* ATCC 13883, *Moraxella catarrhalis* ATCC 23246 and yeast species *Cryptococcus neoformans* ATCC 14116 were selected to represent pathogens related to respiratory conditions. The Gram-positive *Lactobacillus acidophilus* ATCC 314 and *Streptococcus mutans* ATCC 10919 were selected to represent pathogens associated with dental conditions.

Chromobacterium violaceum ATCC 12472 was selected as the biosensor strain for the screening of anti-quorum sensing activity. This micro-organism produces violacein (a purple pigment) which is regulated by the quorum sensing system. By monitoring the violacein content, quorum sensing activity can be monitored.

With the exception of *C. violaceum* ATCC 12472, all cultures were obtained from Davies Diagnostics Pty Ltd (South Africa) and provided by the Department of Pharmacy and Pharmacology, University of the Witwatersrand, South Africa. A waiver for the use of these micro-organisms was granted by the WITS Human Research Ethics Committee (Reference W-CJ-140627-1) (Appendix D). *Chromobacterium violaceum* was supplied by co-supervisor Professor A. Viljoen and the department of Pharmaceutical Sciences, Tshwane University of Technology.

Stock cultures were kept at - 20 ^{o}C. The inoculum was obtained from stock cultures and sub-cultured onto respective media (TSA, LBA) depending on the micro-organisms. The growth media was scrupulously checked for growth before inoculation with culture, to rule out contamination and confirm sterility. Media that showed no growth was suitable for use.

With the exception of *C. violaceum*, all the other test micro-organisms were streaked onto TSA. The TSA plates were incubated at 37 ^{o}C for 24 h for aerobic pathogens were and 37 ^{o}C for 48 h for bacterial and yeast cultures respectively under aerobic conditions. The purity of the cultures was confirmed by pure colonies obtained from the preparation of streak plates.

The Streptococci and *L. acidophilus* were grown under anaerobic conditions using the candle jar method at 37 ºC for 24 h. The purity of these cultures was confirmed by the absence of growth on the streak plates.

Chromobacterium violaceum (ATCC 12472) was cultured in Luria Bertani broth (LBB) (Oxoid) and incubated at 30 °C for 24 h in an orbital shaker incubator (Labcon), shaking at 140 rpm, under aerobic conditions. Thereafter, a streak plate was performed on LBA using inoculum from the broth culture. After incubation the purity of the cultures was confirmed by the pure colonies obtained from the streak plates (Figure 2.3). Thereafter, a single colony from the streak plate was placed into 10 ml of LB broth, from which an inoculum suspension adjusted to approximately 5 x 10^6 CFU ml was prepared. Thereafter, the inoculum suspension (approximately 5 x 10^6 CFU ml) was diluted 1:10 in LB broth to yield a final inoculum suspension of 5 x 10^5 CFU ml which was used for anti-quorum sensing assay.

All micro-organisms were kept viable by sub-culturing weekly in suitable media to maintain a stock culture.

Figure 2.3. Streak plate of *C. violaceum* ATCC 12472.

2.5.3. The minimum inhibitory concentration assay

The antimicrobial activities were determined monthly for both young and mature *E. radiata* leaf samples for a period of one year (January 2014 - December 2014). This was done to evaluate changes in antimicrobial activity due to seasonal variation. To evaluate the efficacy of *E. radiata* essential oil in comparison to commercial *Eucalyptus* oils, the antimicrobial activities of commercial *Eucalyptus* species had to be determined. Both monthly *E. radiata* essential oil samples and commercial *Eucalyptus* essential oils were tested against the same 18 pathogens. Major compounds are known to have a significant role in the biological properties of some essential oils (Freires *et al.*, 2015). Therefore, the antimicrobial activities of the major compounds of the *E. radiata* leaf essential oil were determined independently and in combination (at 1:1 and various ratios relative to their composition within the essential oil). This was done in order to determine a possible correlation between the antimicrobial activity of the essential oil and its chemical composition (structure activity relationship). These major compounds were: 1,8-cineole, (+)-α-terpineol, *S*(–)-limonene and *R*-(+)-limonene. 1,8-Cineole at 98.0% purity (Lot 1054365) was obtained from Fluka. (+)-α-Terpineol at 97.0% purity (Lot 427741/1) was obtained from Fluka. *S*(–)-limonene at 99.0% purity (Lot 054076) was obtained from Fluka and (*R*)-(+)-limonene at 97.0% purity (Lot 301Tl-101) was obtained from Sigma-Aldrich. Limonene is an optically active compound. A previous study by van Vuuren and Viljoen (2007) established differences in the antimicrobial efficacy of the (+) and (–) limonene isomers. Therefore, both limonene forms were evaluated in this study. The antimicrobial activities of the three major compounds 1,8-cineole, α-terpineol and limonene were assessed singularly and in combination. These combinations were tested on the micro-organisms that showed the most susceptibility to *E. radiata* essential oil. Finally, the antimicrobial properties of essential oils used in combination with *E. radiata* leaf essential oil, were also determined independently, in order to gain the necessary data to interpret combination interactions. The broth microdilution method was used to determine the minimum inhibitory concentration (MIC) in order to evaluate the antimicrobial efficacy of essential oils/major compounds in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2012). Using aseptic manipulation, 100 µl sterile broth (TSB or MHB) was transferred into each well of a 96-well micro-titre plate. Stock solutions of 100 µl of the essential oil samples/major compounds, prepared to a concentration of 32

mg/ml in acetone (acetone at 99.5% purity (Lot 3169) was obtained from Associated Chemical Enterprises (Pty) Ltd) were transferred into the first row of the 96-well micro-titre plate. With each test negative controls comprising 100 µl of sterile broth and acetone: water mixture (32 mg/ml) were added into the first row. And positive control comprising conventional antimicrobials (0.01 mg/ml for antibiotics and 0.1 mg/ml for antifungals) were also introduced into the wells of the first row at 100 µl for each test.

Serial dilutions were performed, transferring 100 µl so that the concentration in each consecutive well descending each column was halved. A 0.5 McFarland standard was prepared for each culture suspension (each of the 18 test micro-organisms) in order to avoid variability in results due to inoculum size, and to allow for comparability and reproducibility of the antimicrobial test. A 100 µl of standardized culture suspension (approximately 1x 10^6 CFU/ml) was added to each of well. Each plate was subsequently covered with sterile adhesive microplate sealing tape (NUNC™) in order to prevent evaporation of volatile essential oil components during incubation. Broth used in each assay was left out overnight and checked for turbidity to assess sterility. An inoculum of the standardized culture was streaked on an appropriate agar plate for single colonies to check for purity of culture. Incubation conditions for aerobic pathogens were 37 ^oC for 24 h and 37 ^oC for 48 h for bacterial and yeast cultures respectively. Streptococci and *L. acidophilus* species were grown under anaerobic conditions using the candle jar method.

After incubation, 40 µl of a 0.04% w/v solution of p-Iodonitrotetrazolium chloride indicator (INT) (Sigma-Aldrich) was added to each well of the micro-titre plate. The indicator interacts with viable micro-organisms which causes a colour change from clear to pink. After addition of the INT solution, the micro-titre plates were left at room temperature (25 ^oC) and allowed to develop until a colour change with reference to the culture control column was observed. The MIC was read as the lowest concentration at which no visible growth (no colour change is observed from the plate) after the addition of INT (Figure 2.4). The antimicrobial tests were performed in duplicate, on consecutive days and repetitions were conducted where necessary to maintain accuracy. A review by van Vuuren *et al.* (2008) proposed that for essential oils, an MIC value of 2.00 mg/ml or lower be considered as noteworthy. This was the criterion used in this study for evaluating noteworthy efficacies.

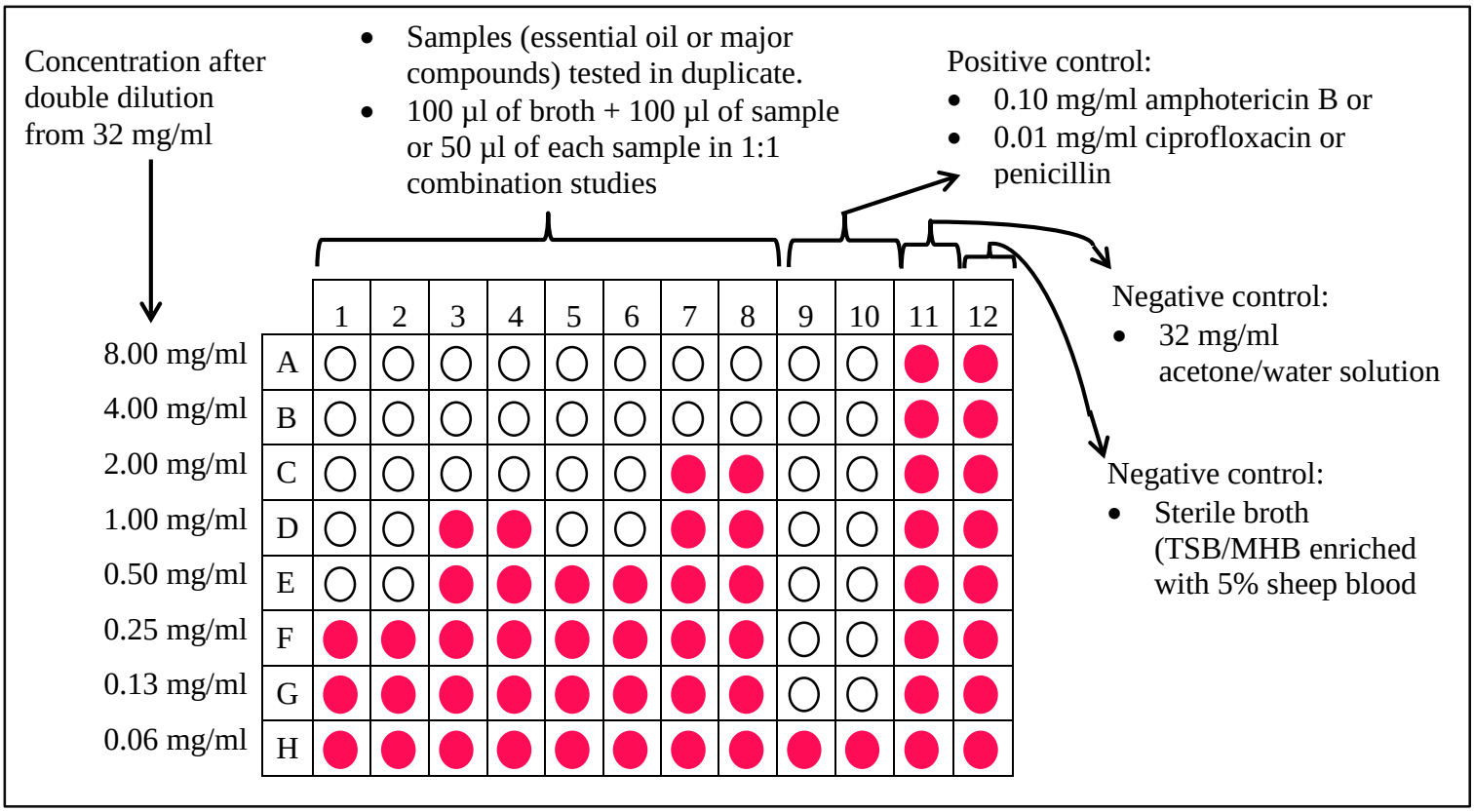

Figure 2.4. Diagram representing an MIC plate. The pink circles represent microbial growth. Inhibition of microbial growth represented by the clear circles. The MIC was read as the lowest concentration at which microbial growth is inhibited, observed in column one and two as 0.50 mg/ml.

2.5.3.1. Minimum inhibitory concentration assay controls

Antimicrobial positive controls were included in the assay to confirm the antimicrobial susceptibility of the micro-organisms. Ciprofloxacin (Sigma-Aldrich) was prepared in sterile distilled water to a 0.01 mg/ml stock concentration for bacteria; with the exceptions of *S. mutans, L. acidophilus, S. pyogenes, S. pneumoniae* and *S. agalactiae*, where penicillin (Sigma-Aldrich) prepared in sterile distilled water to a stock concentration of 0.01 mg/ml. Amphotericin B (Sigma-Aldrich) prepared in sterile distilled water to a 0.10 mg/ml stock concentration was used when testing the yeasts. Negative controls comprising an acetone-water mixture, and broth without antimicrobial agents (culture control) were included in the assay. The acetone-water mixture was included to assess the antimicrobial effect of the solvent. The culture control was included in order to evaluate the ability of the media to

support microbial growth and to serve as a reference for determining when to read MIC results.

2.5.3.2. Interactions at 1:1 combination ratios

The sum of the fractional inhibitory concentration index (FICI) was used to determine the type of interactions between the major compounds of the *E. radiata* leaf essential oil and between the essential oil (*E. radiata*: other essential oil) combinations. For an essential oil demonstrating synergistic activity in combination with *E. radiata* leaf essential oil, further analysis of the interaction of the major compounds from the essential oil, in combination with the major compounds of the *E. radiata* essential oil were studied. Briefly, a volume of 100 µl of sterile MHB was placed into each well of a 96 well micro-titre plate. Thereafter, equal volumes (50 µl of substance A and 50 µl of substance B) of essential oil/compound mixture was introduced into the first row of the micro-titre plate and the serial doubling dilution technique employed. All samples were tested in duplicate. For the determination of interaction of the 1:1 combinations, the sum of the fractional inhibitory concentration (FICI) was calculated and classified as either synergistic (≤ 0.50), additive (> 0.50 - 1.00), indifferent (> 1.00 ≤ 4.00) (van Vuuren *et al.,* 2014). The FICI was calculated according to Equation 2.2;

Equation 2.2a

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

Equation 2.2b

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

*Where (a) is the MIC of one component in the combination and (b) is the MIC of the other component. The sum of the FIC, known as the FIC index is thus calculated as:

Equation 2.2c.

$$FICI = FIC^{(a)} + FIC^{(b)}$$

2.5.3.3. Interactions at relative combination ratios

The FIC method is based on the principle that each test agent is responsible for half of the antimicrobial activity of the combination mixture (Lambert and Lambert, 2003; van Vuuren and Viljoen, 2011). The limitation in the ΣFIC method is that: (a) the two compounds in combination may not have the same dose response and (b) plants do not accumulate compounds at 1:1 ratios (van Vuuren and Viljoen, 2007; van Vuuren and Viljoen, 2011). To account for this, further combination studies were conducted on the major compounds at the relative ratios (mean annual compositional ratio, Chapter 3, Table 3.1) they appeared in the *E. radiata* leaf essential oil. For the relative ratios the 100 µl compound mixtures comprised: 1,8-cineole (84 µl): α-terpineol (16 µl), 1,8-cineole (95 µl): *S*(-)-limonene (5 µl), 1,8-cineole (95 µl): (*R*)-(+)-limonene (5 µl), α-terpineol (77 µl): *S*(-)-limonene (23 µl), α-terpineol (77 µl): (*R*)-(+)-limonene (23 µl). Independent and combination antimicrobial studies were compared to the results of the crude essential oil in order to determine the role of these compounds in the observed antimicrobial activity of this oil.

2.5.4. The antiquorum sensing assay

The anti-quorum sensing activities were determined for *E. radiata* leaf essential oil independently and in combination with *Citrus bergamia* (bergamot), *Lavendula angustifolia* (lavender), *Citrus limon* (lime), *Origanum majorana* (marjoram), *Cymbopogon martinii* (palmarosa), *Mentha piperita* (peppermint), *Rosmarinus officinalis* (rosemary), *Melaleuca alternifolia* (tea tree), *Thymus vulgaris* (thyme), *Citrus aurantifolia* (lemon), *Eugenia caryophyllus* (clove) and *Leptospermum scoparium* (manuka). The major compounds of *E. radiata* leaf essential oil and the major compounds (≥ 10% of essential oil composition) of the essential oil with the most promising antiquorum sensing interaction were further evaluated independently and in combination.

2.5.4.1. Antiquorum sensing broth macrodilution assay

The broth macrodilution (test tube method) method was used to screen for the anti-QS activity of essentials and major compounds, and to determine their minimum quorum sensing

inhibitory concentration (MQSIC). The percentage inhibition of violacein pigment was quantified in treated versus untreated *C. violaceum* ATCC 12472 in order to quantify anti-quorum sensing activity (Ahmad *et al.*, 2014b). Using aseptic manipulation, stock solutions (32 mg/ml in acetone) of essential oil and major compounds respectively, at were added into test tubes containing 5 ml of LB broth to produce concentrations ranging from 0.03 mg/ml to 8.00 mg/ml. This was done in order to evaluate the ability of the essentials/major compounds to inhibit violacein production at various concentrations. A 100 µl of *C. violaceum* ATCC 12472 culture suspension (approximately 5 x 10^6 CFU/ml) was added to each test tube. The test tubes were then incubated at 30 °C for 24 h with agitation at 140 rpm orbital shaker incubator (Labcon). Anti-quorum sensing activity was interpreted based on growth (turbidity) and absence of violacein, a purple pigment (Figure 2.5**)**. After incubation, test tubes were vortexed and the lowest concentration at which no growth (clear) and no pigmentation were observed was interpreted as the MIC. The presence of growth (turbidity) and absence of violacein was interpreted as the minimum quorum sensing inhibitory concentration (MQSIC). The presence of both growth and pigment was interpreted as no anti-QS activity. To confirm the accuracy of the test tube observations, LB agar plates were divided into three parts and streaked with suspensions from test tubes representing the MIC, MQSIC and 0.5 x MQSIC and incubated at 30 °C for 24 h to check for growth.

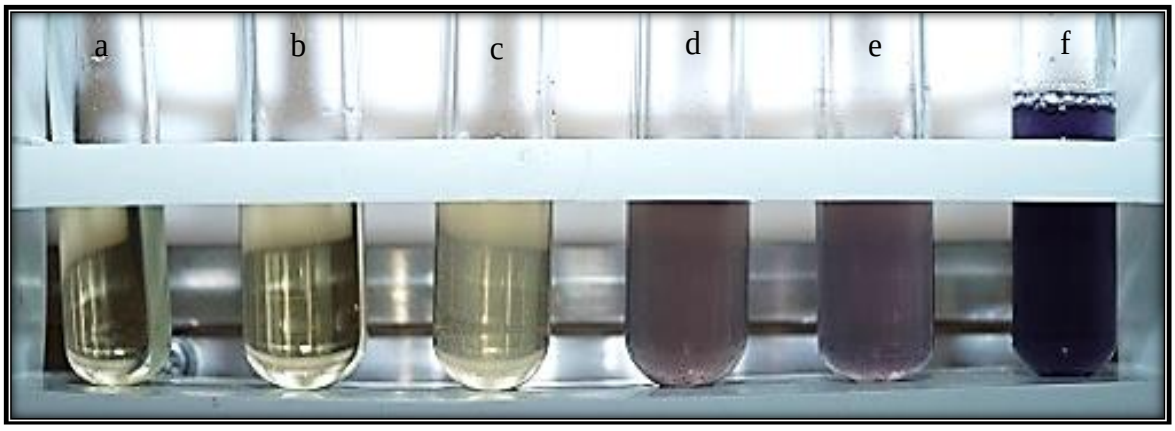

Figure 2.5 Interpretation of antiquorum sensing activity. Test tubes (a) and (b) are clear indicating no antiquorum sensing activity, and no microbial growth. Test tube (c) is turbid which is indicative of active microbial growth and antiquorum sensing activity interpreted as the MQSIC. Test tube (d), (e) and (f) are indicative of the presence of quorum sensing activity due to the presence of violacein (purple pigment).

2.5.4.2. Antiquorum sensing assay controls

Acetone was used as a negative control (solvent control) for each experiment, to ascertain whether the solvent itself has any anti-quorum sensing properties. Vanillin (Sigma) was used as a positive control to confirm the susceptibility of the micro-organism to inhibition of quorum sensing activity. Vanillin is a known inhibitor of quorums sensing activity (Chenia, 2013). A test tube containing untreated *C. violaceum* (ATCC 12472) was added as a control to ensure the ability of the broth to support growth and to also use as a reference point in determining the percentage of violacein inhibition.

2.5.4.3. Determination of percentage violacein detection

After incubation, 1 ml aliquots (of treated or untreated culture suspension) were taken from each test tube and transferred into separate 15 ml conical bottom centrifuge tubes. These were then centrifuged at 5000 rpm for 10 min in an Eppendorf centrifuge 5804 (Merck Millipore) in order to pellet the bacteria containing violacein (Figure 2.6a). The supernatant was discarded. Characteristically, violacein is a water insoluble pigment with antibacterial activity (McClean *et al.,* 1997) and in this study; the violacein was poorly soluble in the solvent of choice, acetone. Therefore, a 100% dimethyl sulfoxide (DMSO) solution was used to solubilise the violacein. DMSO is a widely used in quorum sensing inhibitory assays as a solubilizer with no effects on quorum sensing (Bodini *et al.,* 2009). The remaining bacterial pellet was re-suspended in 1 ml of 100% DMSO solution and each centrifuge tube was vortexed until the pellet was solubilised. After adding DMSO and vortexing, the centrifuge tubes were further centrifuged at 5000 rpm for 7 min (Figure 2.6b). This was done in order to separate bacterial cells from the solution (Figure 2.6c). After centrifugation, 200 µl of the supernatant from each centrifuge tube was placed into two wells of a 96-well micro-titre plate (Figure 2.6d). The test was done in duplicate to ensure accuracy and repetitions were conducted where necessary to maintain accuracy. Violacein was quantified spectrophometrically (Figure 2.6d). The resultant supernatant was used to determine the violacein content by recording the absorbance at OD595 nm, using a FilterMax F5 multi-mode microplate reader (Molecular Devices). The percentage violacein inhibition was calculated using the Equation 2.3:

Equation 2.3; Percentage violacein production = $\frac{\text{control OD595 - test OD595}}{\text{control OD595}} \times 100$

The MQSIC was determined as the lowest concentration at which violacein production was inhibited by ≥ 50 % with reference to the control sample (Alvarez *et al.*, 2012; Ahmad *et al.*, 2014b). Therefore, in this study using *C. violaceum* ATCC 12472, positive anti-QS activity is the concentration at which violacein production is inhibited by ≥ 50%. The percentage violacein inhibition measures the efficacy of the essential oil; the higher the percentage violacein inhibition, the more effective the essential is in inhibiting quorum sensing. The MQSIC measures the potency of the essential oil; the lower the MQSIC, the more potent the essential oil. The desired result would be a combination of a high percentage violacein inhibition and a low MQSIC value.

A
Broth (supernatant) separated from bacterial culture containing violacein (sediment) via centrifugation.

B
Separation of violacein and bacteria via centrifugation.

C
Violacein (supernatant) separated from bacteria (sediment) via centrifugation.

D
200 µl of supernatant, tested in duplicate.

E
Spectrophotometric analysis of violacein content.

Figure 2.6. Determination of percentage violacein inhibition.

2.5.4.4. Interactions at 1:1 combination ratios

The major compounds of the essential oil showing noteworthy antiquorum sensing results in combination with *E. radiata* oil were considered for further analysis with the major compounds of the *E. radiata* l eaf essential oil. Combination studies were undertaken to establish if any synergistic interactions were apparent between essential oils that would lead to increased antiquorum sensing activity. The fractional quorum sensing inhibitory concentration index (FQSICI) was used to investigate the interactions between the 1:1 combinations of *E. radiata* with other test essential oils and between major compounds. Briefly, a volume of 5 ml of sterile LBB was placed into various test tubes plate. To this equal volumes (e.g. 0.63 ml of substance A and 0.63 ml of substance B to create a concentration of 8 mg/ml in test tube one) of essential oil/compound mixture were introduced into each test tube. The total volumes of the mixtures were varied in each test tube order to test various concentrations ranging from 0.03 mg/ml to 8.00 mg/ml. All samples were tested in duplicate and repetitions were performed where necessary. The FQSICI was determined by dividing the MQSIC value of the essential oil in combination by the value of each essential oil in combination independently (Equations 2.4a, Equation 2.4a). These two FQSIC values were then added together to determine the FQSICI (Equation 2.4c). The interpretation of the FQSICI was adapted from the interpretation of the FICI and was interpreted as either synergistic (≤ 0.50), additive (> 0.50 ≤ 1.00), indifferent (> 1.00 ≤ 4.00) or antagonistic (> 4.00) (van Vuuren and Viljoen, 2011; van Vuuren *et al.,* 2014). The ΣFQSIC was calculated according to Equation 2.4:

Equation 2.4a

$$\text{FQSIC}^{(a)} = \frac{\text{MQSIC}^{(a)} \text{ in combination with}^{(b)}}{\text{MQSIC}^{(a)} \text{ independently}}$$

Equation 2.4b

$$\text{FQSIC}^{(b)} = \frac{\text{MQSIC}^{(b)} \text{ in combination with}^{(a)}}{\text{MQSIC}^{(b)} \text{ independently}}$$

*where (a) is the MQSIC of substance A in the combination and (b) is the MQSIC of substance B. The sum of the FQSICI, is thus calculated as:

Equation 2.4 $FQSICI = FQSIC^{(a)} + FQSIC^{(b)}$

2.5.4.5. Interactions at relative combination ratios

The interactions between the major compounds of *E. radiata* essential oil the major compounds of the essential oil showing noteworthy antiquorum sensing results in the 1:1 (*E. radiata*: essential oil) combination were investigated at the relative ratios they would occur within the essential oil mixture. This was achieved by adding the appropriate volume of each compound (from stock concentrations of 32 mg/ml) to 5 ml of broth (Table 2.2). As with the 1:1 combinations, the interactions were determined from the sum of the FQSICI (Equation 2.4).

Table 2.2. Combination of major compounds at ratios relative to their composition within the *E. radiata*: essential oil mixture, and the starting volumes required for a starting concentration of 8.00 mg/ml for antiquorum sensing assay.

Compound mixture	Ratio	Starting volumes in anti-quorum sensing assay (ml)
1,8-cineole: α-terpinene	9:1	1.13 ml: 0.13
(-)-terpinene-4-ol: α-terpinene	8:2	1.00 ml: 0.25
(+)-terpinene-4-ol: α-terpinene	8:2	1.00 ml: 0.25
α-Terpineol: α-terpinene	6:4	0.75 ml: 0.50
γ-terpinene: α-terpinene	7:3	0.88 ml: 0.38
1,8-Cineole: γ-terpinene	8:2	1.00 ml: 0.25
(-)-Terpinene-4-ol: γ-terpinene	6:4	0.75 ml: 0.50
(-)-Terpinene-4-ol: γ-terpinene	6:4	0.75 ml: 0.50
γ-Terpinene: α-terpineol	6:4	0.75 ml: 0.50
1,8-Cineole: (-)-terpinene-4-ol	7:3	0.88 ml: 0.38
1,8-Cineole: (+)-terpinene-4-ol	7:3	0.88 ml: 0.38
(-)-Terpinene-4-ol: α-terpineol	7:3	0.88 ml: 0.38
(-)-Terpinene-4-ol: α-terpineol	7:3	0.88 ml: 0.38
1,8-Cineole: α-terpineol	8:2	1.13 ml: 0.13

CHAPTER 3

Effects of seasonal variation on the yield and chemical composition of young and mature *E. radiata* leaf essential oil

3.1. Introduction

South Africa is a commercial producer and exporter of medicinal *Eucalyptus radiata* essential oil (Coppen and Hone, 1992). Despite the commercial oil production in South Africa, there has been no literature describing the yield patterns and chemical profile (quality) of the material produced and harvested in South Africa. Identifying conditions that result in high essential oil yields, is crucial to optimising the commercial production of the *E. radiata* species.

In previous studies, seasonal variation and leaf age were noted to have a significant effect on the essential oil yield and chemical composition of other *Eucalyptus* species (Silva *et al.*, 2006; Sartorelli *et al.*, 2007; Sefidkon *et al.*, 2010). For example, the amount of essential oil produced and the presence of compounds would vary as a result of seasonal variation and leaf age. These variations may affect the market value. The market value of an essential oil is dependent on the chemical composition of the essential oil (Raut and Karuppayil, 2014). Understanding the effects of these aforementioned factors will give insight into possible ways (e.g. ideal harvest times, ideal growth conditions) to optimize protocols unique to *E. radiata*. Therefore, this Chapter assesses the yield and chemical profile of *E. radiata* young and mature leaf essential oil, over a 12 month period in order to evaluate the variability which may impact on commercial aspects.

3.2. Results and discussion

3.2.1. Essential oil yield

Monthly sampling was undertaken, where essential oil yields and chemical composition were recorded for young and mature leaves, with the exception of the August young leaf sample. The chemical composition of the August young leaf sample demonstrated an anomaly and thus was excluded from the data set (Table 3.1).

Hydro-distillation proved to be an effective method for obtaining essential oil from *Eucalyptus radiata* leaf samples. The essential oil had a distinctive camphorous-peppermint odour, and yielded a pale, milky to clear oil. Distillation yields for all monthly samples are presented in Table 3.1. Hydro-distillation of both young and mature *E. radiata* leaf samples yielded an essential oil range between 0.14% - 4.31% (w/w) throughout the sampling period. The highest oil yields were obtained in summer (December) for both young (3.00%) and mature (4.31%) leaf samples. *Eucalyptus radiata* is noted among the high essential oil yielding species with expected yields estimated in a range between 2.50 - 3.50% (Coppen and Hone, 1992; Pearson, 1993). However, yields may not necessarily always fall within this range. Chisowa (1997) previously noted an essential oil yield as high as 9.00% (Chisowa, 1997) for a Tunisian *E. radiata* sample. A lower yield range between 1.00 - 2.40% was also reported for a South African harvest of *E. radiata* essential oil (Floracopeia.com, 2015)

Figure 3.1 is a graphical presentation of the seasonal variation in the temperature and precipitation patterns of the plant collection area. Figure 3.2 is a graphical presentation of the average essential oil yields from young and mature leaves in different seasons (summer, autumn, winter and spring). A general pattern was observed where high rainfall and high temperate seasons (summer and spring) resulted in higher yields for both young (1.03 - 3.00%) and mature (0.90 - 4.32%) leaf samples. In comparison, low rainfall, low temperate seasons (autumn and winter) resulted in lower yields for both young (0.14 - 0.61%) and mature (0.36 - 2.83%) leaf samples. This similar relationship between oil yield and seasonal variation has been noted in twelve other *Eucalyptus* species (Tsiri *et al.*, 2003; Silva *et al.*, 2006). For all eleven species evaluated by |Silva *et al.*, (2006), seasons characterised by high rainfall and high temperatures resulted in higher oil yields, whereas seasons with low rainfall

resulted in lower oil yields. Tsiri *et al*., (2003) also found higher yields in the hot summer months and lower yields in the cooler winter months for *E. camaldulensis*.

The significance of leaf age on essential oil yield was pronounced during autumn and winter, with mature leaves producing on average two times more oil in comparison to young leaves (Figure 3.2). The pronounced difference in yield between young and mature leaves in autumn and winter may be an indicator of the differences in sensitivity of the two leaves to seasonal variation. The stress of water deficiency and low temperatures caused young leaves to markedly reduce essential oil yield. This indicates that young leaves are more sensitive to the effects of seasonal variation than mature leaves. Therefore, when considering the highest essential oil yield, the best harvesting time for *E. radiata* is during the seasons of summer and spring from mature leaf material.

3.2.2. Chemical composition

The results of the chemical analysis of young and mature *E. radiata* leaf essential oil samples, across a one-year sampling period are presented in Table 3.1. In total, 26 compounds were identified within the 23 essential oil samples accounting for 93.5 - 99.5% of the total oil composition. The major compound found within all samples regardless of seasonal variation and leaf age was 1,8-cineole (41.6 - 79.0%) with an annual mean and standard deviation of 65.7 ± 9.5 (Table 3.1). In addition to 1,8-cineole, other major compounds were α-terpineol (6.7 - 27.4%) with an annual mean and standard deviation of 6.5 ± 2.4 and to a lesser extent, limonene (3.6 - 13.0%) with an annual mean and standard deviation of 12.8 ± 4.4, found at varied concentrations.

The relative ratio of the major and minor compounds varied throughout the annual sampling period. Some of the most significant variance was observed with 1,8-cineole; where autumn (March mature leaf sample) had the lowest composition of 41.6% and summer (December mature leaf sample) had the highest composition of 79.0%. The difference in 1,8-cineole content observed in these two seasons is almost double. The major compounds α-terpineol and limonene also showed approximately four-fold and three-fold differences respectively.

Minor compounds showing notable variances were: geraniol (0.2 - 2.9%), γ-elemene (from trace amounts - 4.6%) and γ-terpinene (from trace amounts - 5.2%).

In conjuction with this study, an untargeted and targeted GC-MS analysis and multivariate analysis was undertaken in order to investigate variation related to leaf maturity and seasonal variation in more detail (Mahumane *et al.,* 2016).

Both approaches/methods demonstrated minimal qualitative changes in the chemical composition of the essential oil due to seasonal variation and leaf age, whereas significant quantitative variation due to leaf age and leaf maturity was demonstrated. This finding supports the observations in this study. In Table 3.1, the type of compounds present within the essential oils of both young and mature leaf samples were relatively the same regardless of seasonal variation. While, the amounts of these compounds varied with each season. For example, high levels of 1,8-cineole were found to be consistent with mature leaves in comparison to younger leaves (Mahumane *et al.*, 2016). This finding is similar to the results depicted in Figure 3.3, where young leaves generally contained less amounts of 1,8-cineole in comparison to mature leaves.

The chemical profiles of *E. radiata* leaf essential oil from various parts of the world have been previously reported (Chapter 1, Table 1.4). Singh (1994) noted 1,8-cineole (74.25%), α-terpineol (11.60%) and limonene (4.50%) as the major compounds in *E. radiata* oil samples from India. Chisowa (1997) noted 1,8-cineole (80.80%), α-terpineol (6.40%) and limonene (3.70%) as the major compounds of a Zambian oil sample. Bendaoud *et al.* (2009) noted 1,8-cineole (69.5%), α-pinene (11.9%) and *trans*-pinocarveol (4.8%) as the major compounds from a Tunisian oil sample. Mulyaningsih *et al.* (2011), reported 1,8-cineole (82.70%), α-terpineol (7.00%) and α-pinene (3.7%) as the major compounds of a German *E. radiata* oil sample. Luís *et al.* (2015) reported limonene (68.51%), α-terpineol (8.60%) and α-terpinyl acetate (6.07%) as the major compounds of the *E. radiata* oil sample from Portugal. 1,8-Cineole was noted as the major compound in majority (7 of 8) of these previous studies on *E. radiata* oil, which is in corroboration with the findings of this study.

Table 3.1. Yield and chemical composition of *Eucalyptus radiata* leaf essential oil for the period January 2014 to December 2014**.**

RRI	Compound	Summer				Autumn						Winter					Spring						Summer		Mean ± standard deviation (SD)
		Jan		Feb		Mar		Apr		May		Jun		Jul		Aug	Sep		Oct		Nov		Dec		
		Young	Mature	Young	Mature	Young	Mature	Young	Mature	Young	Mature	Young	Mature	Young	Mature	Mature	Young	Mature	Young	Mature	Young	Mature	Young	Mature	
	Essential oil yield (%; w/w)	2.64	3.67	1.81	0.90	0.28	0.43	0.61	2.83	0.14	0.36	0.22	1.69	0.14	1.55	1.03	1.03	1.64	1.66	2.44	1.35	2.65	3.00	4.31	1.6 ± 1.2
1016	α-Pinene	2.3	2.2	1.2	2.0	3.6	5.1	2.5	2.2	2.3	2.6	0.4	3.1	2.1	2.6	2.6	1.2	1.3	1.5	2.5	3.8	2.5	2.3	1.2	2.3 ± 1.0
1019	α-Thujene	0.2	0.2	0.1	0.2	0.1	0.3	0.2	0.2	0.2	0.1	tr	0.2	tr	0.2	0.3	0.1	0.1	0.2	tr	0.2	0.2	0.1	0.1	0.2 ± 0.1
1104	β-Pinene	0.8	0.7	0.5	0.6	0.6	1.2	0.7	0.6	0.8	0.7	0.3	0.8	0.7	0.7	0.9	0.5	0.6	0.6	0.7	1.0	0.8	1.1	tr	0.7 ± 0.2
1117	Sabinene	1.4	1.0	1.1	0.8	0.7	1.1	0.8	0.7	0.8	0.9	0.5	0.9	1.4	0.7	0.7	0.7	0.9	0.7	0.7	0.6	1.2	0.7	0.5	0.8 ± 0.3
1159	Myrcene	2.0	1.7	1.4	1.9	2.2	3.3	1.6	1.3	2.0	1.7	0.8	2.0	1.7	1.5	1.9	1.0	1.1	1.2	1.5	3.1	1.3	3.3	tr	1.8 ± 0.7
1174	α-Terpinene	0.1	0.2	0.2	0.2	0.3	0.3	0.2	0.2	0.3	0.2	0.1	0.3	0.2	0.3	0.3	0.2	0.1	0.2	tr	0.4	0.3	0.7	0.1	0.2 ± 0.1
1194	**Limonene[a]**	**6.3**	**6.5**	**4.6**	**4.4**	**5.5**	**12.8**	**6.4**	**6.3**	**8.3**	**6.5**	**3.6**	**7.4**	**6.1**	**6.4**	**6.5**	**4.2**	**4.6**	**5.1**	**5.9**	**9.4**	**6.7**	**13.0**	**3.7**	**6.5 ± 2.4**
1202	**1.8-Cineole[a]**	**66.9**	**68.6**	**68.0**	**66.0**	**63.8**	**41.6**	**66.6**	**66.3**	**56.2**	**67.2**	**52.4**	**73.0**	**71.4**	**75.1**	**66.0**	**77.3**	**73.6**	**69.2**	**72.0**	**53.1**	**69.3**	**47.7**	**79.0**	**65.7 ± 9.5**
1242	γ-Terpinene	0.2	0.4	0.3	0.4	0.5	0.7	0.4	0.5	0.7	0.5	0.3	0.6	0.3	0.5	5.2	0.3	0.2	0.4	0.5	0.7	0.5	tr	tr	0.7 ± 1.0
1250	(E)-β-Ocimene	0.4	0.3	0.3	0.1	1.3	0.6	0.3	0.3	0.4	0.3	0.4	0.5	0.3	0.3	tr	0.2	0.3	0.5	tr	0.7	0.3	1.3	0.2	0.4 ± 0.3
1270	*p*-Cymene	0.6	0.5	0.1	0.6	0.2	0.8	0.2	0.3	0.3	0.3	0.1	0.3	0.4	0.3	0.3	0.3	0.1	0.3	tr	0.2	0.1	0.1	0.1	0.3 ± 0.2
1281	Terpinolene	0.1	0.1	0.1	0.1	0.2	0.2	0.1	0.1	0.2	0.1	tr	0.1	0.1	0.1	tr	0.1	tr	0.2	tr	tr	0.1	0.3	0.1	0.1 ± 0.1
1382	Z-3-Hex-en-1-ol	tr	Tr	tr	tr	tr	tr	0.1	0.1	tr	0.1	tr	tr	tr	tr	tr	tr	tr	tr	tr	0.1	0.4	tr	tr	0.1 ± 0.1
1541	Linalool	0.5	0.4	0.7	0.3	0.3	0.3	0.5	0.5	0.5	0.5	0.6	0.2	0.3	0.3	0.4	0.2	0.5	0.4	tr	0.4	0.3	tr	0.2	0.4 ± 0.1
1563	Trans-p-menth-2-en-1-ol	0.2	0.2	0.1	0.6	0.1	0.1	0.1	0.1	0.1	0.1	0.3	tr	0.1	0.1	tr	0.2	0.2	0.2	tr	tr	0.1	0.2	tr	0.2 ± 0.1
1602	Terpinene-4-ol	1.1	1.2	0.9	1.7	0.5	1.3	1.4	1.7	2.0	1.4	1.9	0.2	0.9	1.1	1.3	1.1	1.0	1.3	1.7	1.5	0.1	2.4	0.7	1.2 ± 0.6
1674	γ-Terpineol	0.2	0.2	0.3	0.3	0.3	0.3	0.2	0.2	0.3	0.2	0.4	0.1	0.2	0.1	0.1	0.2	0.2	0.2	tr	0.3	tr	0.3	0.2	0.2 ± 0.1
1689	Neral	0.1	0.2	0.2	0.5	0.1	0.1	0.2	0.2	0.3	0.2	0.4	tr	0.1	0.1	tr	0.1	0.2	0.2	tr	0.3	0.2	tr	0.2	0.2 ± 0.1
1701	**α-Terpineol[a]**	**12.6**	**11.0**	**15.0**	**13.6**	**6.7**	**13.7**	**12.9**	**14.4**	**18.8**	**12.3**	**27.4**	**7.0**	**10.7**	**7.6**	**10.4**	**9.5**	**11.9**	**13.1**	**10.5**	**17.0**	**11.4**	**16.4**	**10.1**	**12.8 ± 4.4**
1740	Geranial	0.5	0.3	0.4	0.7	0.2	0.2	0.2	0.3	0.4	0.2	0.6	tr	0.2	0.1	0.3	0.1	0.4	0.4	tr	tr	0.2	tr	0.1	0.3 ± 0.2
1743	γ-Elemene	0.2	0.4	0.3	0.5	4.6	4.1	0.5	0.6	0.9	0.5	0.4	0.2	0.2	0.2	tr	0.1	0.1	0.2	tr	0.3	0.3	0.3	0.1	0.7 ± 1.2
1822	Geraniol	1.8	1.0	2.7	0.2	0.7	3.6	1.7	1.8	2.1	1.4	6.3	0.9	1.5	0.9	1.5	1.1	1.4	1.6	1.0	2.5	1.3	2.9	1.2	1.8 ± 1.3
2141	Spathulenol	tr	0.1	tr	0.1	0.1	0.7	0.1	0.1	0.2	0.1	0.1	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	0.1	tr	0.2 ± 0.2
2181	γ-Eudesmol	0.1	0.2	0.1	0.3	0.4	0.7	0.1	0.1	0.2	0.2	0.2	0.1	tr	0.1	0.1	0.1	tr	0.1	tr	0.2	0.1	tr	tr	0.2 ± 0.2

2235	α-Eudesmol	0.1	0.3	0.1	0.4	0.4	1.0	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.1	tr	0.1	tr	0.1	tr	tr	0.1	tr	tr	0.2 ± 0.2
2245	β-Eudesmol	0.1	0.4	0.1	0.5	0.4	1.2	0.2	0.1	0.2	0.3	0.3	0.1	0.1	0.1	tr	0.1	tr	0.1	tr	tr	0.1	0.3	tr	0.3 ± 0.3
	Total area percentage (%)	98.8	98.3	98.8	97.0	93.8	95.3	98.4	99.4	98.7	98.8	98.0	98.1	99.1	99.5	98.8	99.0	98.8	98.0	97.0	95.8	97.9	93.5	97.8	

Bold = Major compounds; tr = trace amounts (< 0.1% composition).

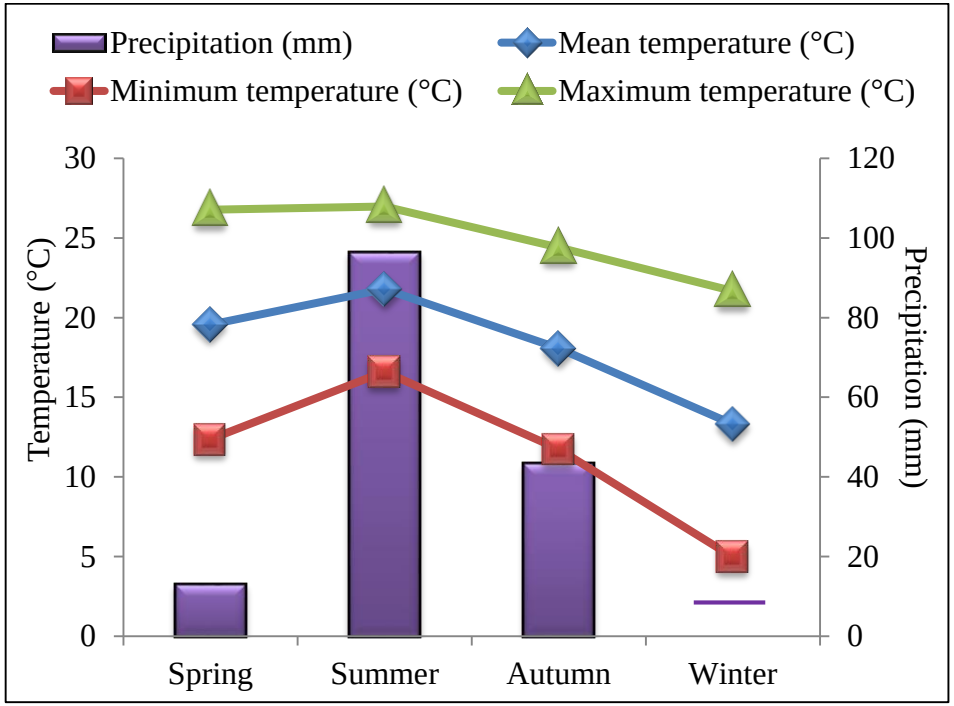

Figure 3.1. Temperature and precipation patterns of the sampling region in the various seasons of spring (September, October and November), summer (December, January and February), autumn (March, April and May) and winter (June, July and August) ClimatView/worldclimate/TCC [WWW Document] (n.d); Historical weather for 2014 in Polokwane, South Africa [WWW Document] (2014).

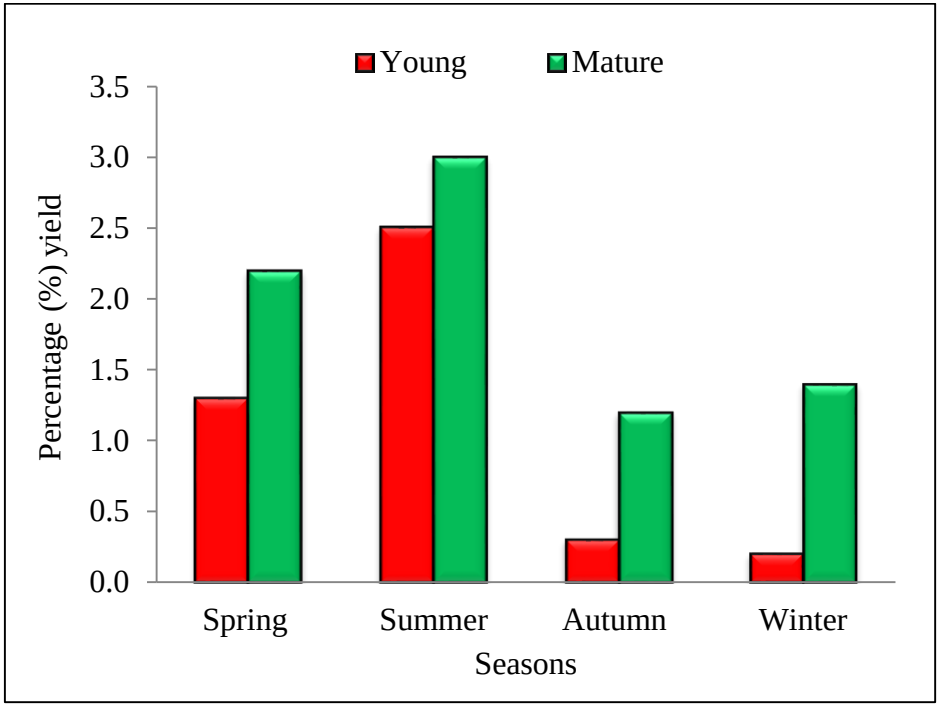

Figure 3.2. Average essential oil yield of young and mature *Eucalyptus radiata* leaf samples in various seasons.

The only exception was with the *E radiata* sample reported by Luís *et al.* (2015). This sample was not only devoid of the major compound 1,8-cineole, but contained limonene as the major compound. This shows that besides quantitative variation, qualitative differences in the chemical composition can also occur within the *E. radiata* species. These differences can be attributed to the differences in environmental conditions and genetic factors, since the essential oil seeds and location were different (Coppen, 2002; Bendaoud *et al.*, 2009; Mulyaningsih *et al.*, 2011). These differences in major compounds between the South African harvested *E. radiata* sample and that of Luís *et al.* (2015) can also be taken as evidence of the existence of *E. radiata* chemotypes.

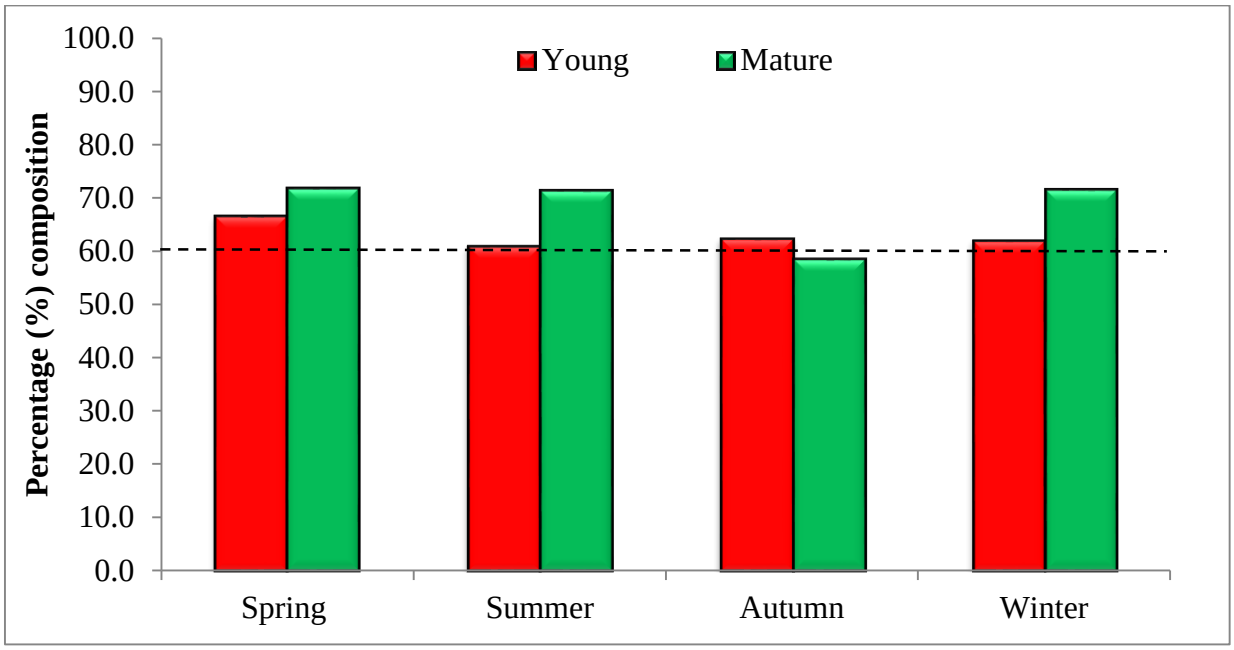

Figure 3.3 Average seasonal 1,8-cineole composition of young and mature *Eucalyptus radiata* leaf essential oil samples. Line (---) indicating minimum 1,8-cineole content required for commercial feasibility.

There are many references on the changes in essential oil composition due to seasonal variation among *Eucalyptus* species (Coppen and Hone, 1992; Coppen, 2002; Silva *et al.*, 2006; Bendaoud *et al.*, 2009; Derwich *et al*, 2009; Juan *et al.*, 2011; Sefidkon *et al.*, 2010; Silva *et al.*, 2011). One example of this is where the major compound of *E. cinerea*, 1,8-cineole varied between 60.70% in summer and 83.61% in winter. The other major compound α-terpinyl acetate, varied 20.44% summer and 5.38% in winter Silva *et al.*, (2011). However,

there have been no previous studies on the effects of seasonal variation on the chemical composition of the essential oil to compare results with until this study.

3.2.3. Comparison to other *Eucalyptus* species

The 1,8-cineole rich oil is the most commercially important oil *Eucalyptus* essential oil, primarily used in pharmaceutical preparations (Muchori *et al.*, 1997). Therefore, commercial potential was assessed by comparing the 1,8-cineole content of the medicinal *Eucalyptus radiata* essential oil to other commercial *Eucalyptus* essential oil samples.

Analysis of commercial essential oils sourced from different *Eucalyptus* species, showed quantitative differences in 1,8-cineole content. In order of highest to lowest, the 1,8-cineole content ranked: *E. globulus* (90.00%) > *E. smithii* (84.72%) > *E. radiata* (comm) (65.45%) > *E. dives* (8.96%) > *E. citriodora* (0.46%) (Table 3.2). In order to qualify *Eucalyptus* essential oil for pharmaceutical use, the oil needs to fulfill the following criteria: the oil should contain a high 1,8-cineole content, coupled with the absence of the compounds isovaleraldehyde and α-phellandrene and β-phellandrene (Chisowa, 1997; Singh, 1994). Phellandrene has been reported to possess weak mutagenic and carcinogenic properties, while isoveraldehyde is a compound with an unpleasant odour, associated with irritation of the throat when it is inhaled resulting in coughing (Pages *et al.*, 1990; Coppen, 2002; Guba, 2009). For commercial viability, medicinal *Eucalyptus* oil is required to contain at least 60.00% - 65.00% 1,8-cineole (Coppen and Agriculture Organization of the United Nations, 1995). According to the above mentioned criteria, only essential oils from *Eucalyptus globulus* (90.00%) > *Eucalyptus smithii* (84.72%) > *Eucalyptus radiata*-comm (65.45%) and the Tzaneen harvested *E. radiata* leaf essential oil samples theoretically qualify as medicinal type oils. *Eucalyptus globulus* (90.00%) is the most globally used medicinal *Eucalyptus* oil source, and it showed the highest cineole content. This is followed by *E. smithii* (84.72%), the primary source of medicinal *Eucalyptus* essential oil in South Africa (Pech, 2006). After *E. smithii*, *Eucalyptus radiata* (65.45%) is the secondary source of medicinal *Eucalyptus* in South Africa.

Eucalyptus radiata was shown to produce medicinal (1,8-cineole rich) type essential oil (Table 3.1). All *E. radiata* leaf essential oil test samples in this study were devoid of the

undesirable compounds (phellandrene and isovaleradehyde), making this oil suitable for medicinal purposes.

Table 3.2. Chemical composition of *Eucalyptus* essential oils.

Compound	***Eucalyptus* species**					
	radiata*	***radiata*-comm**	***globulus***	***citriodora***	***smithii***	***dives***
α-Pinene	2.3	2.28	2.30	*	8.29	0.71
α-Thujene	0.1	0.26	0.01	*	*	2.75
β-Pinene	0.7	0.62	0.44	0.60	0.37	*
Sabinene	0.9	1.28	0.00	*	*	0.22
Myrcene	1.7	1.43	0.02	*	*	21.37
α-Terpinene	0.2	*	0.10	*	*	1.45
Limonene	6.5	3.24	*****	*****	*****	1.14
1.8-Cineole	**65.7**	**65.45**	**90.00**	0.46	**84.72**	8.96
Citronellal		*	*	**70.90**	*	*
γ-Terpinene	0.6	0.45	2.16	*	*	0.94
(E)-β-Ocimene	0.4	0.26	0.09	*	*	0.45
p-Cymene	0.3	0.93	3.60	*	*	4.90
Terpinolene	0.1	*	0.14	*	4.09	2.32
Z-3-Hex-en-1-ol	0.0	*	0.00	*	0.17	*
Linalool	0.4	0.53	0.06	*	*	1.19
Trans-p-menth-2-en-1-ol	0.1	0.19	0.00	*	*	0.51
Terpinene-4-ol	1.2	1.93	0.00	*	0.32	5.12
γ-Terpineol	0.2	0.29	0.01	*	0.32	0.21
Neral	0.2	0.56	0.00	*	*	*
α-Terpineol[a]	12.8	15.05	0.06	*	1.57	4.03
Geranial	0.3	1.31	*	*	*	**41.16**
γ-Elemene	0.7	0.22	*	*	*	*
Geraniol	1.8	1.01	0.00	*	*	*
Spathulenol	0.1	*	0.00	1.29	*	*
γ-Eudesmol	0.1	*	0.01	5.66	*	*
α-Eudesmol	0.2	*	*	*	0.15	*
β-Eudesmol	0.2	*	*	*	*	*
Total area percentage (%)	97.74	97.29	99.00	78.90	100.00	97.44

* = compound not identified; * = Mean percentage composition of *E. radiata* leaf essential oil included for comparative purposes (Table 3.1).

In terms of commercial feasibility (minimum 60.00% - 65.00% 1,8-cineole content), 74.00% of the tested samples fulfill this requirement, the majority of which were obtained in summer and spring. (Table 3.1, Figure 3.3).

The 1,8-cineole content of the *E. radiata* leaf essential oil samples, determined from the mean and standard deviation was noted at 65.70% ± 9.50 (Table 3.1). This is similar to the 65.4% 1,8-cineole content of its commercially acquired counterpart (*E. radiata*-comm essential oil). Some *E. radiata* oil samples had 1,8-cineole composition higher than the commercially acquired counterpart (e.g. December mature leaf sample 79.00%), Table 3.1. This is an indicator for the potential to produce higher amounts of a 1,8-cineole which may stimulate interest in its commercial use. Last but not least, *E. radiata* oil is reportedly one of the most widely used *Eucalyptus* oils in aromatherapy, and is often preferred to the popular *E. globulus* due to its pleasant odour (Rhind, 2012; Mulyaningsih *et al.*, 2011). Commercially, its pleasant aroma can attract higher prices (e.g. offers an alternative *Eucalyptus* oil with a more pleasant aroma).

3.3. Overview of Chapter 3

- Mature leaves harvested in seasons characterised by high rainfall and high temperatures (summer and spring) resulted in higher oil yields for the *E. radiata* species.

- Young and mature *E radiata* leaf essential oil samples from the same site demonstrated consistent qualitative composition across the 12 month sampling period. Only quantitative variation was observed.

- The *E. radiata* leaf essential oil sample displayed a chemical composition comparable to its commercial counterpart (*E. radiata*-comm).

- *Eucalyptus radiata* showed potential for use as a commercial medicinal *Eucalyptus* oil source with a mean 1,8-cineole content of 65.7% ± 9.5.

CHAPTER 4

Antimicrobial activity of *E. radiata* leaf essential oil

4.1. Introduction

The antimicrobial efficacy of *E. radiata* essential oil has previously been investigated against selected pathogens (Lis-Balchin and Deans, 1997; Inouye *et al.*, 2001; Takarada *et al.*, 2004; Bendaoud *et al.*, 2009; Mulyaningsih *et al.*, 2011; Luís *et al.*, 2015). However, the majority of these studies were conducted using the disc diffusion method, while limited attention has been given to the preferred quantitative MIC method. Therefore, a more up-dated, more reliable analysis using the quantitative MIC method is warranted.

Furthermore, there is a lack of comparable antimicrobial data available for this species. For instance, details of the chemical composition (chemical profile) of *E. radiata* essential oil samples are often not reported in previous studies (Takarada *et al.*, 2004; Lis-Balchin and Deans, 1997). This makes comparison of antimicrobial activity between *E. radiata* oil samples challenging. Bendaoud *et al.* (2009) and Mulyaningsih *et al.* (2009) for example, reported chemical composition along with antimicrobial activity, these results are also not comparable as different antimicrobial assays were used. In addition, these previous studies evaluated different pathogens: i.e. food related pathogens (Lis-Balchin and Deans, 1997), dental pathogens (Takarada *et al.*, 2004), plant pathogens (Bendaoud *et al.*, 2009), drug resistant pathogens (Mulyaningsih *et al.*, 2011) and problematic Gram-negative bacteria associated with nosocomial infections (Luís *et al.*, 2015).

In view of these previous gaps in literature, this Chapter presents a comprehensive and in-depth antimicrobial analysis of *E. radiata* essential oils obtained monthly from young and mature leaves. Analysis is done in correlation with the chemical composition results presented in Chapter 3, Table 3.1.

Considering the interest of the *Eucalyptus* essential oil industry in South Africa, there is great potential for research and commercial development of this understudied species. Should commercial interest arise, the antimicrobial activity of *E. radiata* leaf essential oil will be compared to the efficacy of commercially acquired essential oils from other popular *Eucalyptus* species, such as *E. globulus* and *E. citriodora* (Mulyaningsih *et al.*, 2011). Knowledge of antimicrobial activity across a one-year sampling period independently and in comparison to other commercial *Eucalyptus* essential oils, can be combined to evaluate the feasibility of the potential application of *E. radiata* young and mature leaf oil in the essential oil market.

4.2. Results and discussion

4.2.1. Antimicrobial efficacy of *Eucalyptus radiata* leaf essential oils

Figures 4.1, 4.2, 4.3 and 4.4 are graphical representations of the antimicrobial activity of the 23 monthly *E. radiata* leaf essential oil samples investigated against 18 micro-organisms. Noteworthy antimicrobial activity is considered at $\leq$ 2.00 mg/ml for essential oils (van Vuuren, 2008). Therefore, noteworthy activity was observed throughout the sampling period from monthly samples of both young and mature leaf oils for 11 of the 18 test pathogens (Figures 4.1, 4.2, 4.3 and 4.4**).**

The most susceptible micro-organisms were the Streptococci and *L. acidophilus*; particularly *S. mutans* with an MIC range of 0.25 - 1.00 mg/ml and *L. acidophilus* with an MIC range of 0.19 - 1.75 mg/ml throughout the sampling period (Figure 4.1). The noteworthy activity of the *E. radiata* oil against dental pathogens correlates with previous findings on cariogenic and periodontopathic micro-organisms, reported by Takarada *et al.* (2004). Takarada *et al.* (2004) also noted the antimicrobial activity of the *E. radiata* oil against *S. mutans* (1.00% MIC), *S. sobrinus* (1.00% MIC), *P. gingivalis* (0.25 - 0.50% MIC), *A. actinomycetemcomitans* (0.50 % MIC), and *F. nucleatum* (0.13 - 0.25% MIC). Antimicrobial activity against *L. acidophilus* has not been previously reported, therefore, this study is the first to note the efficacy of ~~the~~ *E. radiata* leaf essential oil against this particular pathogen. The efficacy of the *E. radiata* oil against dental pathogens *S. mutans* and *L. acidophilus* demonstrates that this oil may be of

importance in the treatment of dental conditions, as indicated in Figure 4.1. *Streptococcus mutans* and *L. acidophilus* both showed susceptibility at 0.5 mg/ml to both young and mature leaf oils, consistently throughout summer and spring (Figure, 4.1). In contrast, variation in microbial susceptibility to the inhibitory effects of the essential oils was observed in autumn and winter, particularly with *L. acidophilus*. The lowest and highest MIC values were noted in autumn (March) and winter respectively. Moreover, between the two pathogens, *L. acidophilus* was the least susceptible to the inhibitory effects of the essential oil in the autumn / winter seasons (Figure 4.1). A similar pattern in antimicrobial activity was observed between young and mature leaves (i.e. same MIC values in summer/winter, and varied antimicrobial efficacy in autumn/winter seasons).

Predominantly noteworthy antimicrobial activity was noted against the pathogens associated with gastrointestinal/food related infections from both young and mature leaves throughout the sampling period. Among the pathogens associated with gastrointestinal/food related conditions, *L. monocytogenes* and *B. cereus* were the most susceptible, with MIC values of 0.25 - 1.00 mg/ml and 0.25 - 2.00 mg/ml respectively throughout the sampling period (Figure 4.2). The MIC values were between 2.00 - 3.00 mg/ml for *E. coli* and 1.00 - 4.00 mg/ml for *S. typhimurium* in this study across the sampling period. In comparison, much lower MIC values of 16 µl/ml and 32 µl/ml, for *E. coli* and *S. typhimurium* respectively have been reported in literature (Luís *et al.*, 2015). The *E. radiata* essential oil sample presents as a different chemotype (limonene-rich) to the 1,8-cineole-rich sample in this study (Chapter 1, Table 1.1). Differences in activities can be attributed to the sum of the compound interactions between the different compounds within the two *E. radiata* essential oil samples. With regards to the other pathogens, there were no previous studies found (using the quantitative MIC method) reporting on the activity of *E. radiata* leaf essential oil against *S. sonnei*, *B. cereus* and *L. monocytogenes* to compare results with. No notable variation in antimicrobial activity was observed between young and mature leaf samples across the sampling period. The noteworthy antimicrobial activity against the food related micro-organisms in this study indicates that the *E. radiata* leaf essential oil may have potential for the application as a preservative in the food industry.

The MIC values of the wound / skin related pathogens demonstrated that *P. aeruginosa* was the most susceptible micro-organism with an MIC value ranging between 0.50 - 1.75 mg/ml throughout the sampling period (Figure 4.3). In comparison, Luís *et al.* (2015) also noted efficacy of the oil at an MIC value of 32 µl/ml, confirming the efficacy of *E. radiata* leaf essential oil against *P. aeruginosa*. A literature review by Burt (2004) noted that *P. aeruginosa* is the micro-organism least sensitive to the antimicrobial action of essential oils. In this study however, *P. aeruginosa* was found to be one of the most sensitive micro-organisms when compared to the Gram-positive bacteria (*S. aureus*, methicillin-resistant *S. aureus* and *E. faecalis*) and yeast (*C. albicans*) associated with wound/skin infections (Figure 4.3).

The emergence of resistant micro-organisms such as MRSA present a problem for the control of micro-organisms in infectious conditions in terms of treatment availability (Edris, 2007). It is interesting to note that the methicillin-resistant *S. aureus* (MRSA) strain was relatively more susceptible in comparison to the non-resistant *S. aureus* strain (Figure 4.3). In comparison Mulyaningsih *et al.* (2011) previously noted poor to moderate (≥ 4 mg/ml) antimicrobial activity of *E. radiata* oil against MRSA. These differences may be attributed to the differences in chemical composition between the two essential oils. The drug resistant MRSA was most susceptible particularly in the summer (November and December months). Interestingly, during these two months, the chemical composition of the essential oil varied significantly between young and mature leaves. For instance, the major compounds 1,8-cineole, limonene and α-terpineol ranged between 47.40 - 79.00%, 10.10 - 17.00% and 3.70 - 13.00% respectively. The variation in the ratios of the major compounds was too large and non-uninform to identify a clear chemical compositional variation pattern to explain the observed antimicrobial activity against MRSA. However, it is evident that the unique compound ratios in November and December resulted in increased antimicrobial activity against MRSA.

Traditionally, *Eucalyptus* oils were used in Aboriginal medicine for the healing of wounds and associated infections (Takahashi *et al.*, 2004; Sartorelli *et al.*, 2007). Among its many uses, *E. radiata* oil is used in the promotion of wound healing and treatment of skin conditions such as acne (Higley and Higley, 1998; Balz *et al.*, 1999). The noteworthy

antimicrobial activity of the essential oil against pathogens associated with wound/skin infections shows there is *in vitro* rationale behind its use for these conditions.

Among the pathogens associated with respiratory infections: *S. agalactiae* (0.19 - 1.00 mg/ml), *S. pneumoniae* (0.19 - 1.00 mg/ml), *S. pyogenes* (0.25 - 2.00 mg/ml), and *C. neoformans* (0.25 - 2.00 mg/ml) had the highest susceptibility to the essential oil (Figure 4.4). This was followed by *K. pneumoniae* (2.00 - 3.00 mg/ml) with *M. catarrhalis* (2.00 - 4.00 mg/ml) showing the weakest activity. *Eucalyptus radiata* has been termed "the oil of respiration" and known for its efficacy in in the treatment of respiratory conditions (Rose and Earle 1996; Mulyaningsih *et al.*, 2011). This finding serves as *in vitro* rationale for its use in respiratory conditions. Moreover, these results highlight the potential application of the *E. radiata* oil in conditions associated with these respiratory pathogens, especially *S. agalactiae* and *S. pneumoniae*. Very little variation in antimicrobial activity was observed between monthly young and mature leaf oil samples across the sampling period.

4.2.2. Comparison to other *Eucalyptus* species

A comparative analysis of the antimicrobial activity of the *E. radiata* oil with other commercially acquired *Eucalyptus* essential oils such as *E. globulus, E. radiata*-comm, *E. dives, E. smithii* and *E. camaldulensis* is presented in Table 4.1. The various *Eucalyptus* essential oils exhibited predominantly noteworthy activity against the 18 test micro-organisms (Table 4.1). The MIC values ranged between 0.25 - 2.00 mg/ml for *E. smithii* and *E. dives*; 0.50 - 3.00 mg/ml for *E. citriodora*; 0.25 - 4.00 mg/ml for *E. radiata, E. globulus, E. camaldulensis* respectively and 0.50 - 4.00 mg/ml for *E. radiata*-comm. These MIC ranges show that the *E. radiata* leaf essential oil has comparable efficacy to commercial *Eucalyptus* oils, including *E. globulus* and *E. smithii*. *Eucalyptus globulus* is the most popular source of medicinal *Eucalyptus* essential oil (Bajaj, 1995; Shankaranarayana *et al.*, 2006; Jones *et al.*, 2007). *Eucalyptus smithii* is the primary source of medicinal *Eucalyptus* essential oil in South Africa. Both these essential oils contain considerably higher 1,8-cineole content in comparison to *E. radiata* oil (Chapter 3, Table 3.2), however, these essential oils showed similar antimicrobial activity to the *E. radiata* essential oil sample (Table 4.1).

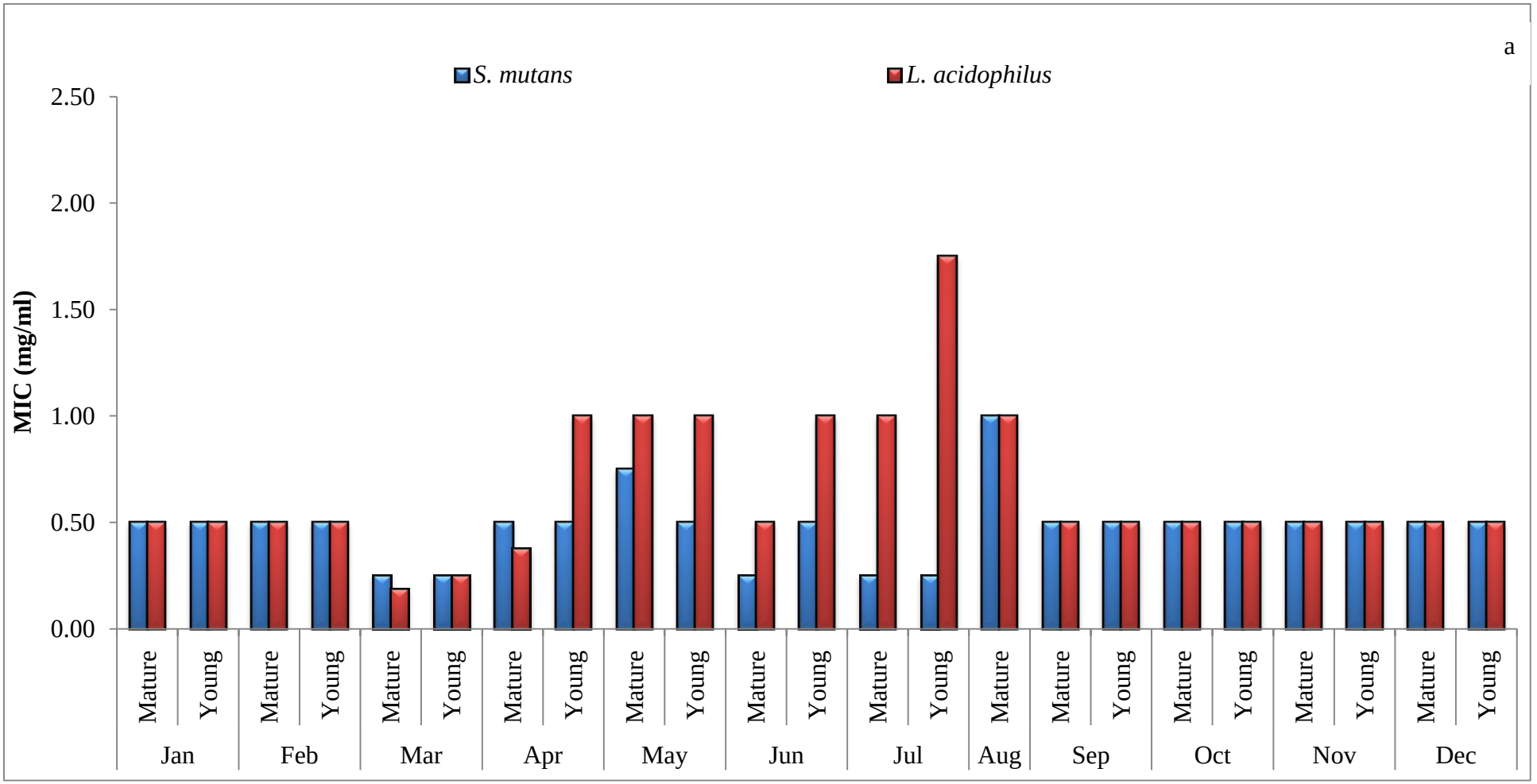

Figure 4.1. Antimicrobial activity (mean MIC expressed in mg/ml) of monthly young and mature *E. radiata* leaf essential oil samples across a one year sampling period, against micro-organisms associated with dental infections.

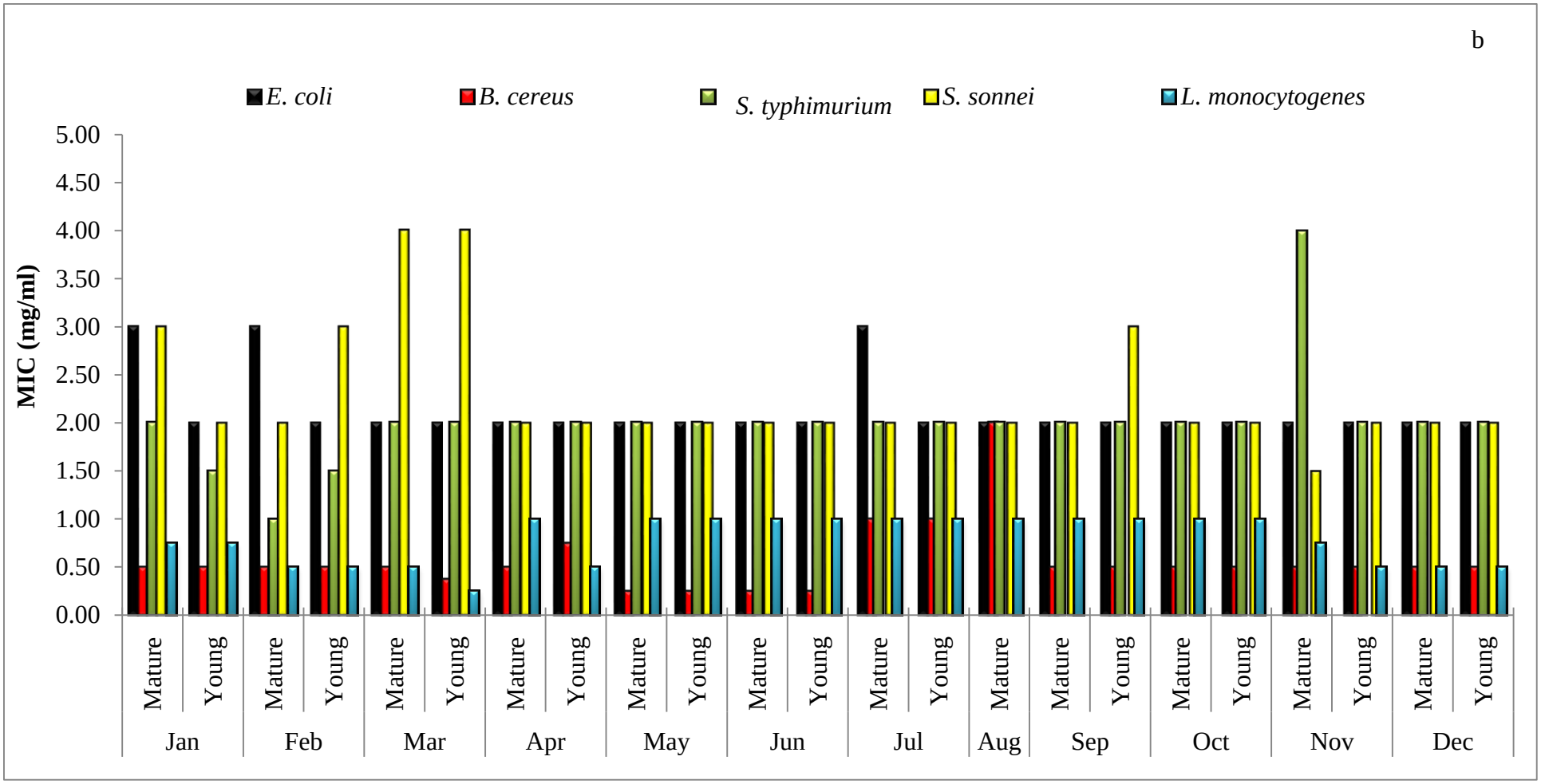

Figure 4.2. Antimicrobial activity (mean MIC expressed in mg/ml) of monthly young and mature *E. radiata* leaf essential oil samples across a one year sampling period, against micro-organisms associated with gastrointestinal infections.

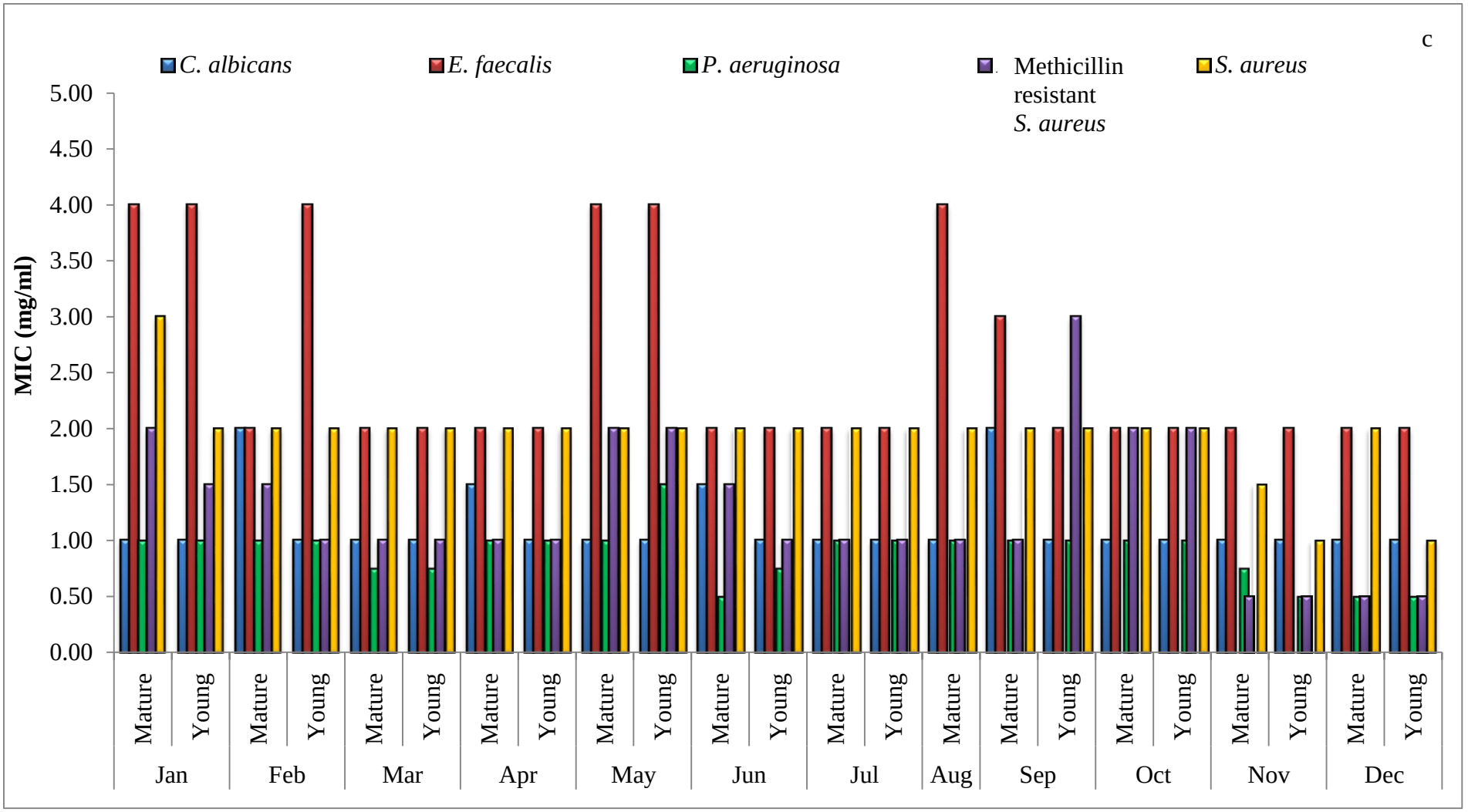

Figure 4.3. Antimicrobial activity (mean MIC expressed in mg/ml) of monthly young and mature *E. radiata* leaf essential oil samples across a one year sampling period, against micro-organisms associated with wound infections.

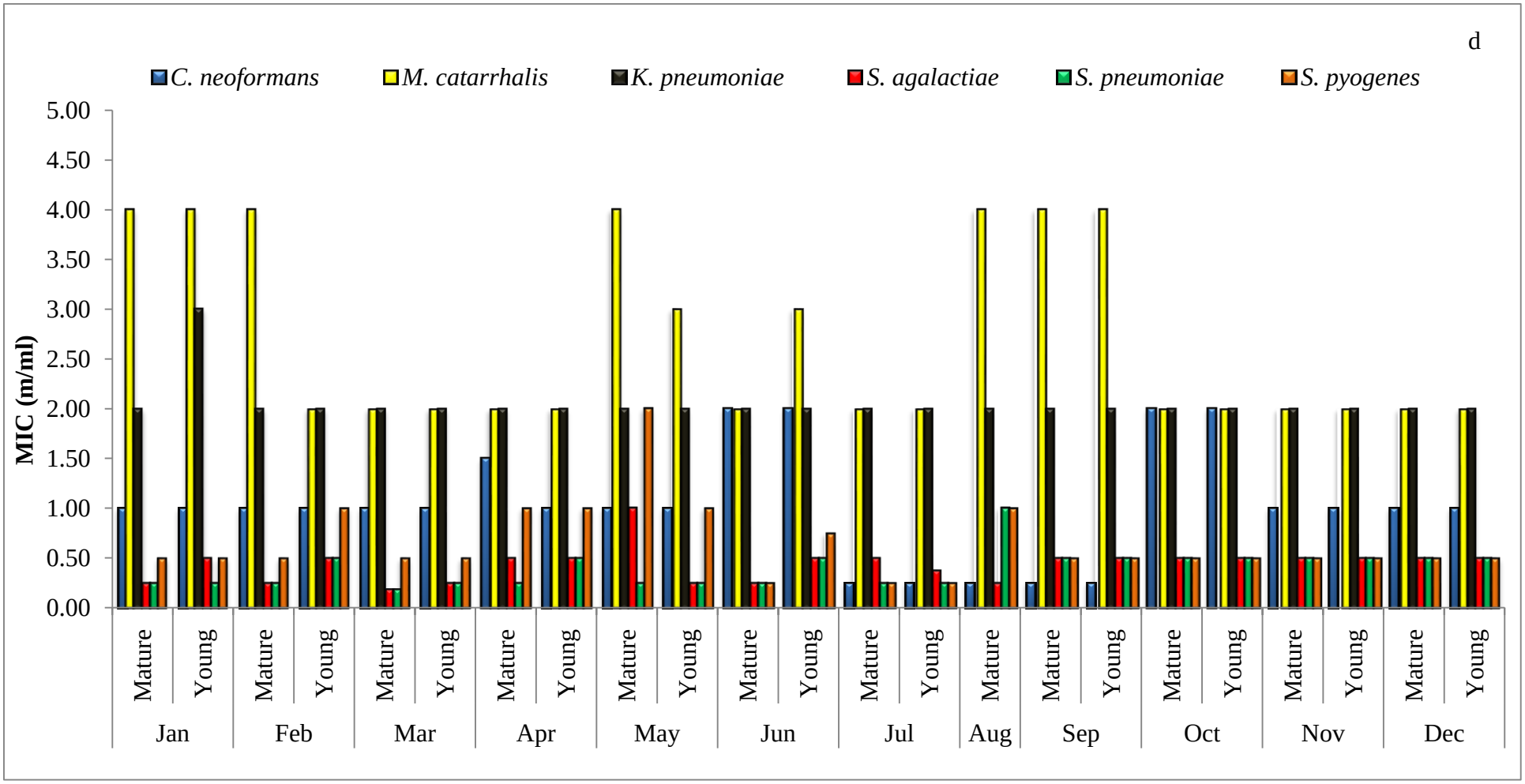

Figure 4.4. Antimicrobial activity (mean MIC expressed in mg/ml) of monthly young and mature *E. radiata* leaf essential oil samples across a one year sampling period, against micro-organisms associated with respiratory infections.

In general, an antimicrobial pattern similar to that observed with *E. radiata* oil was noted for other *Eucalyptus* essential oils; where the Streptococci (associated with respiratory and dental infections) and *L. acidophilus* were the most susceptible micro-organisms (Table 4.1). The noteworthy activity against *S. mutans* and *L. acidophilus* indicates that these *Eucalyptus* essential oils may be of commercial importance in the treatment of dental conditions and general improvement of oral health. *Eucalyptus* essential oil has been used for oral care for many years (Shishir *et al.,* 2011). Duke and Castle (2001) noted the efficacy of a *Eucalyptus* tincture in killing bacteria associated with cavities. *Eucalyptus* essential oil is reportedly effective when applied undiluted for tooth pain, and chewing the young leaves aids in promoting dental hygiene and prevention of halitosis (Duke and Castle, 2001; Shishir *et al.*, 2011). The use of the *Eucalyptus* essential oil highlights its role in dentistry. This is further supported by the incorporation of *Eucalyptus* essential oil and its major compound 1,8-cineole in commercial products like; Colgate Herbal™ toothpaste (Colgate-Palmolive company, South Africa) and Listerine™ (Johnson and Johnson (*Pty*) *Ltd*, South Africa). The activity of *E. radiata* oil against *S. pneumoniae* at 0.25 mg/ml, was the lowest MIC value in comparison to all other *Eucalyptus* essential oils including its commercial counterpart (*E. radiata*-comm). *Eucalyptus* essential oils are commonly used in the treatment of respiratory conditions (Salari *et al.*, 2006; Sartorelli *et al.*, 2007). *Eucalyptus radiata* oil in particular is reportedly useful in the treatment of respiratory tract (Rose and Earle, 1996; Mulyaningsih *et al.*, 2011). The good activity against the Streptococci associated with respiratory conditions provides a rationale for the application of these oils in the treatment of respiratory conditions.

Eucalyptus globulus (0.75 mg/ml) and *E. citriodora* (1.00 mg/ml) displayed relatively higher activity against MRSA in comparison to *E. radiata* essential oil (MIC 2.00 mg/ml). In comparison, Mulyaningsih *et al.* (2011) reported *E. citriodora* to have the highest activity against MRSA while *E. globulus* ($2.00 \geq 4.00$ mg/ml) and *E. radiata* ($4.00 \geq 4.00$ mg/ml) leaf showed similar activities against MRSA. Sherry *et al.* (2001) noted the efficacy of *E. globulus* leaf oils in formulations against MRSA infection of the bone. The noteworthy activities of other *Eucalyptus* essential oils against MRSA show that other species such as *E. radiata* have potential for use as alternative or additional sources in such formulations. This is beneficial as *E. radiata* is often preferred to the *E. globulus* oil due to its pleasant aroma (Mulyaningsih *et*

al., 2011). Having alternative options introduces opportunities of variety (such as different scents) in future commercial formulations.

Table 4.1. Mean (n= ≥ 2) MIC values in mg/ml of different *Eucalyptus* leaf essential oils.

Pathogens	***Eucalyptus* species**							
	***radiata*‡**	***radiata*-comm***	***globulus*‡**	***camaldulensis*‡**	***citriodora***	***smithii****	***dives****	**Control**
B. cereus	**0.50**	**1.50**	**0.25**	**0.25**	**1.00**	**2.00**	**1.00**	$0.039e^{-3}$
C. albicans	**1.00**	**1.00**	**1.00**	**0.50**	**1.00**	**1.00**	**1.00**	$3.125e^{-3}$
C. neoformans	**1.00**	**1.00**	**1.00**	**0.50**	**1.00**	**1.00**	**1.00**	$6.250e^{-3}$
E. faecalis	**2.00**	3.00	**1.50**	**2.00**	**2.00**	**2.00**	**2.00**	$0.625\ e^{-3}$
E. coli	**2.00**	**2.00**	**2.00**	**2.00**	**2.00**	**2.00**	**2.00**	$0.625e^{-3}$
K. pneumoniae	**2.00**	**2.00**	**2.00**	**2.00**	**2.00**	**2.00**	**2.00**	$0.039\ e^{-3}$
L. acidophilus	**0.50**	**1.00**	**1.00**	**0.38**	**0.75**	**1.00**	**1.00**	$0.310e^{-3}$
L. monocytogenes	**0.75**	**1.00**	**0.50**	**0.50**	**0.50**	**1.00**	**1.00**	$0.625e^{-3}$
Methicillin-resistant *S. aureus*	**2.00**	**1.00**	**0.75**	**0.50**	**1.00**	**2.00**	**1.00**	$1.250e^{-3}$
S. aureus	**2.00**	**2.00**	**2.00**	**0.50**	**1.00**	**2.00**	**2.00**	$0.625\ e^{-3}$
M. catarrhalis	4.00	4.00	4.00	4.00	**2.00**	**2.00**	**2.00**	$0.313e^{-3}$
P. aeruginosa	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**	**2.00**	**1.00**	$0.313e^{-3}$
S. typhimurium	**2.00**	**2.00**	4.00	**2.00**	3.00	**2.00**	**2.00**	$0.039e^{-3}$
S. sonnei	3.00	**1.50**	3.00	**2.00**	**1.00**	**2.00**	**1.50**	$0.625\ e^{-3}$
S. agalactiae	**0.25**	**0.50**	**0.25**	**0.25**	**0.75**	**0.25**	**0.25**	$0.310e^{-3}$
S. mutans	**0.50**	**0.50**	**0.25**	**0.25**	**0.50**	**0.50**	**0.25**	$0.160e^{-3}$
S. pneumoniae	**0.25**	**1.00**	**2.00**	**1.00**	**1.00**	**2.00**	**1.00**	$1.250e^{-3}$
S. pyogenes	**0.50**	**1.00**	**0.50**	**0.50**	**1.00**	**0.50**	**0.50**	$0.310e^{-3}$

Noteworthy activity is in bold. *Commercially acquired essential oils; ‡Laboratory acquired essential oils. Ciprofloxacin was used as the control for bacteria excluding Streptococci and *L. acidophilus* where penicillin was used as the control. Amphotericin B was used as the control for the yeast.

Overall, *E. radiata* exhibited similar antimicrobial activity to the commercial grade *E. radiata*-comm essential oil and other essential oils from other *Eucalyptu*s species (Table 4.1). Even though *E. globulus* is the most documented and most commonly used species, and *E. smithii* is the primary source of medicinal *Eucalyptus* oil in South Africa; equal credibility should be given to the *E. radiata* essential oil study sample as based on how well it compares in antimicrobial efficacy to these commercial essential oils.

4.3. Overview of Chapter 4

- *Eucalyptus radiata* leaf essential oil displayed noteworthy antimicrobial activity against a broad-spectrum of pathogens associated with dental, gastrointestinal/food related, wound and respiratory conditions.

- Among the 18 tested pathogens, the best overall activity was noted against the dental pathogens *S. mutans* (0.25 - 1.00 mg/ml) and *L. acidophilus* (0.19 - 1.75 mg/ml).

- *Listeria monocytogenes* (0.25 - 1.00 mg/ml) was the most susceptible of the pathogens associated with gastrointestinal/food related conditions.

- *Pseudomonas aeruginosa* (1.00 - 2.00 mg/ml) was the micro-organism most susceptible to the inhibitory effects of the *E. radiata* essential oil among the pathogens associated with wound/skin related conditions.

- *Streptococcus agalactiae* (0.19 - 1.00 mg/ml) and *S. pneumoniae* (0.19 - 1.00 mg/ml) were the micro-organisms most susceptible to the inhibitory effects of *E. radiata* essential oil among the pathogens associated with respiratory conditions.

- The *E. radiata* test sample displayed similar antimicrobial activity to its commercial counterpart (*E. radiata*-comm) and other commercial *Eucalyptus* essential oils.

CHAPTER 5

The role of major compounds on the antimicrobial properties of *E. radiata* leaf essential oil

5.1. Introduction

The aim of this chapter is to evaluate the antimicrobial properties of the major compounds of the *E. radiata* leaf essential oil and determine a correlation between chemical composition and the observed antimicrobial activity of the essential oil. Burt (2004) previously noted that the antimicrobial activity of the essential oil is related to the proportions of its constituents. The biological properties of an essential oil may be determined by the major compounds (Freires *et al.*, 2015). Therefore, the major compounds (compounds representing the highest proportion of the oil) were selected for antimicrobial analysis.

As noted in Chapter 3, Table 3.1, *E. radiata* oil is composed of several volatile constituents, where compound interactions are bound to occur. Therefore, major compounds will be evaluated independently to identify bioactive compounds and at various ratios in order to determine possible synergistic interactions that may have potential implications on the overall antimicrobial activity.

5.2. Results

5.2.1. The antimicrobial activity of major compounds

The major compounds of the *E. radiata* leaf essential oil were tested against the pathogens that showed the most susceptibility to crude leaf essential oil, which were the Streptococci

and *L. acidophilus*. In this study, the stereochemistry of limonene was not identified from the chemical analysis; therefore, both limonene enantiomers were tested.

All of the major compounds displayed noteworthy antimicrobial activity against the five test pathogens (Table 5.1). The MIC value was 2.00 mg/ml for 1,8-cineole against all five pathogens. The MIC value ranged between 0.75 - 1.00 mg/ml for α-terpineol, 0.25 - 0.75 mg/ml for *S*-(-)-limonene and 0.25 - 0.63 mg/ml for *R*-(+)-limonene (Table 5.1). These results show that for the *E. radiata* leaf essential oil, the compound present in the highest proportion (1,8-cineole), is not necessary the most active compound. Instead, limonene (both (+) and (-) isomers) presented as the most antimicrobially active compound from the three major compounds. Similarly, even though 1,8-cineole was the major compound in an *E. radiata* essential oil from a previous study, α-terpineol was identified as the principle compound contributing to the antimicrobial activity of the essential oil (Inouye *et al.*, 2001).

In contrast to previous reports (Lis-Balchin, 2002; van Vuuren and Viljoen, 2007), stereochemistry had no influence on the antimicrobial activity of limonene. Both limonene enantiomers displayed similar antimicrobial activity, against all five test micro-organisms (Table 5.1). Silva *et al.* (2012) also reported enantiomers of other compounds to have the same antimicrobial activities.

5.2.2 Major compounds in 1:1 combinations

The ΣFIC values of the 1:1 combinations ranged from 0.31 - 1.67 (Table 5.1), corresponding to synergistic, additive and indifferent (predominant) effects (van Vuuren *et al.,* 2014). In this study, combinations containing limonene as one of the compounds generally resulted in an enhanced antimicrobial activity (synergistic or additive interaction). The 1:1 combination of α-terpineol and *S*-(-)-limonene resulted in the highest number of synergistic interactions, with synergy observed for four of the five tested micro-organisms (*L. acidophilus, S. agalactiae, S. mutans* and *S. pneumoniae*), Table 5.1. The 1:1 combinations of 1,8-cineole the with *S*-(-)-limonene and 1,8-cineole the with *R*-(+)-limonene predominantly showed non-interactive results.

The antimicrobial activity of the 1:1 combinations were lower than the activity of at least one of the paired substances independently. This confirms the presence of interactions between these essential compounds. Moreover, the range of resultant interactions (synergistic, additive and indifferent) show that, depending on the type of compounds combined, antimicrobial activity may be altered.

5.2.3. Major compounds combined in relative ratios

As shown in Table 5.1, the individual compounds do not have the same dose response (compounds have different activities at the same concentration), and in reality, plants do not accumulate compounds in 1:1 ratios (Chapter 3, Table 3.1). To account for this, the major compounds were combined at the relative ratios (mean annual compositional ratio, (Chapter 3, Table 3.1) in which they appeared within the crude *E. radiata* leaf essential.

The FICI values of the relative ratio combinations ranged between 0.38 - 4.50 (Table 5.1). In comparison to the 1:1 combinations, significantly less synergistic interactions resulted, and an antagonistic (4.50) interaction was observed. The antagonistic interaction resulted from a combination of 1,8-cineole and *R*-(+)-limonene against *S. pyogenes*. It is interesting to note that when 1,8-cineole and *R*-(+)-limonene were combined at 1:1 ratio against *S. pyogenes* an indifferent interaction was observed, whereas at the relative compositional ratios antagonism is observed. As with the 1:1 combinations, limonene still has a notable effect on the activity. The general pattern observed was that, combinations containing limonene in higher proportion exhibited better antimicrobial activity than combinations in which limonene is present in lower proportion.

5.2.4. Essential oil versus pure compounds

While limonene (both enantiomers) was the only compound to show relatively better activity against the whole essential oil against *L acidophilus*, *S. pyogenes* and *S. mutans* (Table 5.1) However, overall, the individual compounds had similar activity to the MIC values of the whole (crude) essential oil (Table 5.1). This may provide some premise to the notion that the antimicrobial properties of an essential oil may be correlated to the major compounds (Freires

et al., 2015). However, these compounds do not exist in isolation, within *E. radiata* essential oil (Chapter 3, Table 3.1). These major compounds exist in combinations within the essential oil, therefore numerous interactions occur between them and with minor compounds. *Eucalyptus radiata* oil contains a variety of other biologically active minor compounds such as; myrcene, linalool, β-pinene, α-pinene, terpinolene to name a few (Derwich *et al.*, 2009; Park *et al.*, 2012; Freires *et al.*, 2015). The sum of these interactions is what determines the antimicrobial properties of an essential oil. Therefore, research into the antimicrobial and modulatory effects of minor compounds independently and in combination with these major compounds is recommended in order to gain a more holistic understanding of the antimicrobial activity of the essential oil.

The variation in activity of compounds tested independently and in combination results confirms the presence of interactions between compounds within the oil, and a relationship between chemical composition and antimicrobial activity. The combination results indicate that given the correct compositional ratios, antimicrobial efficacy could be altered (enhanced or reduced). In this study, limonene has the strongest influence on the potency of the antimicrobial activity of the combination.

Table 5.1. Mean MIC (mg/ml) for the major compounds independently and in combination with FICI (in brackets), determined for 1:1 combinations and combinations at various ratios.

Compound	***Lactobacillus acidophilus***	***Streptococcus agalactiae***	***Streptococcus mutans***	***Streptococcus pneumoniae***	***Streptococcus pyogenes***
*Eucalyptus radiata**	0.50	0.25	0.50	0.25	0.50
Independent compounds					
1,8-Cineole	2.00	2.00	2.00	2.00	2.00
α-Terpineol	0.88	1.00	0.75	1.00	0.75
S-(-)-Limonene	0.38	0.75	0.38	0.50	0.25
R-(+)-Limonene	0.38	0.63	0.25	0.50	0.25
1:1 Combinations					
1,8-cineole : α-Terpineol	1.00 (0.82)	1.5 (1.13)	1.00 (0.92)	1.5 (1.13)	1.00 (0.92)
1,8-Cineole : *S*-(-)-Limonene	0.50 (0.79)	0.50 (**0.46**)	0.25 (**0.40**)	0.25 (**0.31**)	0.50 (1.13)
1,8-Cineole: *R*-(+)-Limonene	0.50 (0.79)	0.50 (0.53)	0.25 (0.56)	0.25 (**0.31**)	0.50 (1.13)
α-Terpineole : *S*-(-)-Limonene	0.25 (**0.48**)	0.38 (**0.44**)	0.25 (**0.50**)	0.25 (**0.38**)	0.25 (0.67)
α-Terpineole : *R*-(+)-Limonene	0.25 (**0.48**)	0.25 (**0.33**)	0.25 (0.67)	0.25 (**0.38**)	0.25 (0.67)
S-(-)-Limonene : *R*-	0.25 (0.67)	0.25 (**0.37**)	0.50 (1.67)	0.25 (**0.50**)	0.25 (1.00)

Compound	*Lactobacillus acidophilus*	*Streptococcus agalactiae*	*Streptococcus mutans*	*Streptococcus pneumoniae*	*Streptococcus pyogenes*
(+)-Limonene					
Various ratios (relative to essential oil composition in Chapter 3, Table 3.1)					
1,8-Cineole : α-Terpineol	2.00 (1.64)	2.00 (1.50)	1.00 (1.83)	1.00 (0.75)	1.00 (0.92)
1,8-Cineole : *S*-(-)-Limonene	1.00 (2.25)	2.00 (1.83)	1.50 (2.35)	1.00 (1.25)	1.00 (2.25)
1,8-Cineole: *R*-(+)-Limonene	1.00 (1.57)	2.00 (2.09)	1.00 (2.25)	1.00 (1.25)	2.00 (4.50)
α-Terpineole : *S*-(-)-Limonene	0.50 (0.94)	1.00 (1.17)	0.25 (**0.50**)	0.25 (**0.38**)	0.50 (1.33)
α-Terpineole : *R*-(+)-Limonene	0.50 (0.94)	1.00 (1.29)	0.25 (0.67)	0.25 (**0.38**)	0.50 (1.33)
Control (Penicillin)	0.31×10^{-3}	0.31×10^{-3}	0.16×10^{-3}	1.25×10^{-3}	0.31×10^{-3}

Bold = Synergistic activity; (values in brackets) = FICI.

5.3. Overview of Chapter 5

- All three major compounds displayed noteworthy antimicrobial activity against *L. acidophilus*, *S. agalactiae*, *S. mutans*, *S. pneumoniae* and *S. pyogenes*

- Limonene (both enantiomers) was the most active of the major compounds.

- Limonene was the compound with the best overall effect when combined at a 1:1 ratio, mixtures containing predominantly limonene resulted in synergistic or additive interactions.

- Combinations at 1:1 ratios resulted in 13 synergistic, 17 additive, zero indifferent and no antagonistic interactions.

- Significantly less synergistic interactions (a total of three) were observed from combinations at various ratios in comparison to 1:1 ratio combinations.

- Antagonism was noted with the combination of 1,8-cineole and *R*-(+)-limonene against *S. pyogenes*.

CHAPTER 6

The antimicrobial properties of *E. radiata* in combination with selected essential oils

6.1. Introduction

Essential oils are often used in combination with the hope of increasing the therapeutic effect, by taking advantage of synergistic effects (van Vuuren and Viljoen, 2011; Bassole and Juliani, 2012). *Eucalyptus radiata* essential oil is used in combination with other essential oils for the treatment of a variety of conditions (Chapter 1, Table 1.3*)*. According to Guba (2009), *E. radiata* oil can be used in 'synergy' with *E. caryophyllus* (clove), *M. alternifolia* (tea tree), *A. fragrans* (fragonia), *C. martini* (palmarosa), *O. majorana* (sweet marjoram), and *Thymus vulgaris* (thyme) for enhanced antibacterial properties. However, no *in vitro* studies have been found to confirm any synergy, or the effects of *E. radiata* essential oil in combination with any other essential oil. This Chapter will evaluate the antimicrobial interactions of these essential oils in 1:1 combinations with *E. radiata* essential.

6.2. Results

The purpose of screening other essential oils was to evaluate the changes in antimicrobial activity when combined with *E. radiata* leaf essential oil. Therefore, instead of the large number of pathogens (18) investigated for *E. radiata* essential oil in Chapter 4, screening was restricted to three pathogens. These micro-organisms were: *S. aureus* (Gram-positive bacteria), *P. aeruginosa* (Gram-negative bacteria) and *C. albicans* (yeast). These micro-organisms represent each class of micro-organisms likely to be associated with the various conditions displayed in Chapter 1, Table 1.3. Results of the antimicrobial activity of the essential oils independently and in combination with *E. radiata* are summarised in Table 6.1.

6.2.1. The antimicrobial activity of other essential oils

Predominantly noteworthy activity was observed against all three pathogens. The MIC values ranged between 0.50 - 4.00 mg/ml against *S. aureus* (Table 6.1). Of the 12 essential oils tested, 66.67% demonstrated noteworthy activity against *S. aureus. Eugenia caryophyllus* (clove) with an average MIC of 0.50 mg/ml showed the best overall antimicrobial activity against *S. aureus*.

The MIC values ranged between 1.00 - 3.00 mg/ml against *P. aeruginosa.* (Table 6.1). Of the 12 essential oils tested, 91.67% demonstrated noteworthy activity against *P. aeruginosa. Melaleuca alternifolia* (tea tree), *R. officinalis* (rosemary) and *T. vulgaris* (thyme) each with an average MIC of 1.00 mg/ml having the lowest antimicrobial activity against *P. aeruginosa*.

Predominantly noteworthy activity was observed against all three pathogens. The MIC values ranged between 0.50 - 2.00 mg/ml for *C. albicans.* (Table 6.1). Of the 12 essential oils tested, 100% demonstrated noteworthy activity against *C. albicans. Origamun majorana* (marjoram) with an average MIC of 0.50 mg/ml and *T. vulgaris* (thyme) with and average MIC of 0.50 mg/ml showed the best overall antimicrobial activity against *C. albicans*. The essential oils of *O. majorana* (marjoram) and *T. vulgaris* (thyme) are reportedly used in the treatment of skin conditions associated with *C. albicans* infection and athletes foot due to their reported fungicidal activity (Lawless, 2013).

Burt (2004) and Oussalah *et al.* (2007) had noted that essential oils tend to be more susceptible towards Gram-positive bacteria. In this study however, no obvious differences in susceptibility to the inhibitory effects of the essential oils was found between the Gram-positive *S. aureus* and the Gram-negative *P. aeruginosa*.

6.2.2. Essential oils in a 1:1 combination with *E. radiata*

Overall combinations showed a predominantly indifferent interactive profile. The FICI values ranged between 0.75 - 1.25 against *S. aureus*. Of the 12 essential oil combinations tested, 66.67% additive and 33.33% indifferent interactions were observed activity against *S. aureus*.

With regards to *P. aeruginosa*, the FICI values ranged from 0.75 - 3.00 comprising 91.67% indifferent interactions and only 8.33% additive interactions. With *C. albicans*, the FICI values ranged from 1.00 - 3.00 comprising 83.33% indifferent interactions and only 16.67% additive interactions. No synergistic or antagonistic interactions were noted for all combinations against any pathogen.

In this study essential oils were combined with *E. radiata* to evaluate whether, combination with *E. radiata* would result in an improvement in antimicrobial activity. According to the combination results, this *E. radiata* essential oil does not have an enhancing effect on the antimicrobial activities of the 12 essential oils studied when combined at a 1:1 ratio. To the best of our knowledge, this is the first study to cite the antimicrobial effects of the popular *E. radiata* essential oil in combination with other essential oils. These results serve as preliminary data on the effect of *E. radiata* on the antimicrobial activity of individual essential oils. However, these results are not to be taken as a property of *E. radiata* essential oil in essential oil blends. In aroma therapeutic practice, *E radiata* is seldom combined with one essential oil, but is rather incorporated within a blend comprising more than one other essential oil (Chapter 1, Table 1.3). Generally the *E. radiata*: other essential oil ratio is less likely to be a 1:1 ratio.

According to our literature review, the *E radiata* essential oil blends are mostly used for respiratory conditions (e.g. such as sinus infection, sore throat, bronchitis), followed by wound/skin related conditions (Chapter 1, Table 1.3). Each essential oil presents with a wide range of therapeutic properties (e.g. antimicrobial, anti-fungal, expectorant) (Kovac, 2009; Lawless, 2013). Furthermore, essential oils are combined with the belief that the properties of the essential oil blend will reflect the properties of each of the essential oils in the mixture. *Eucalyptus radiata* is well known for its efficacy in the treatment of respiratory conditions (Rose and Earle 1996; Coppen, 2002; Mulyaningsih *et al.*, 2011). Therefore, although the *E. radiata* oil combinations did not result in synergistic interactions, this therapeutic property (treatment of respiratory conditions) of the *E. radiata* essential oil may serve as the rationale for its incorporation in the respiratory blends. In addition, *E. radiata* is reportedly favoured by aromatherapists due to its pleasant fragrance which may also attribute to its selection and use in essential oil combinations (Guba, 2009; Mulyaningsih *et al.*, 2011).

Essential oil combinations are used in hope of increasing the therapeutic outcome. This can be achieved by taking advantage of synergistic interactions between essential oils. From an antimicrobial perspective, the mechanism by which synergy is achieved with essential oil blends. In a combination, each essential oil contributes bioactive compounds with unique target sites on the micro-organism. It is likely that the mode of action by which synergy is achieved in combinations is through the inhibition of several target sites on the micro-organism (Burt, 2004; Bassole and Juliani, 2012). Contrary to the goal of aromatherapy blending, according to literature, essential oil combinations do not necessarily exhibit all the properties of the individual essential oils (Bassole and Juliani, 2012; de Rapper *et al.*, 2013). But rather; the properties of the essential oil blend reflects the sum of the interactions between the essential oils. According to literature, the combination of essential oils with similar compounds results in additive instead of synergistic effects, which has been attributed to the presence of phenolic compounds (Lambert *et al.*, 2001; de Azeredo *et al.*, 2011; Bassole and Juliani, 2012). The explanation for this is that, similar compounds will have a similar or the same mechanism of action, resulting in an additive antimicrobial action. This can be used to explain the observations made in this study. For example, the major compounds of the majority of the essential oils were terpenes compounds such; as verbenone (*Rosmarinus officinalis*), 1,8-cineole (*Origanum marjorana*), limonene (*Citrus bergamia, Citrus aurantifolia* and *Lavandula angustifolia*) for example. Combining these with *E. radiata* leaf essential oil which also comprises a terpene 1,8-cineole as a major compound resulted in additive interactions, most frequently against *S. aureus*.

Table 6.1. Antimicrobial activity of essential oils independently and in a 1:1 combination with *Eucalyptus radiata* leaf essential oil against *S. aureus*, *P. aeruginosa* and *C. albicans*.

Essential oil	Independent essential oils			1:1 combination with *E. radiata* essential oil		
	***S. aureus* (ATCC 25923)**	***P. aeruginosa* (ATCC 27853)**	***C. albicans* (ATCC 10231)**	***S. aureus* (ATCC 25923)**	***P. aeruginosa* (ATCC 27853)**	***C. albicans* (ATCC 10231)**
Eucalyptus radiata	**2.00**	**1.00**	**1.00**	*	*	*
Citrus bergamia (bergamot)	4.00	**2.00**	**2.00**	3.00 (1.13)	4.00 (3.00)	**2.00** (1.50)
Eugenia caryophyllus (clove)	**0.50**	**2.00**	**1.00**	**1.00** (1.25)	**2.00** (1.50)	**2.00** (2.00)
Lavendula angustifolia (lavender)	**2.00**	**2.00**	**1.00**	**2.00** (1.00)	**2.00** (1.50)	**2.00** (2.00)
Citrus aurantifolia (lemon)	**2.00**	**2.00**	**1.00**	**2.00** (1.00)	**2.00** (1.50)	**1.00** (2.00)
Citrus limon (lime)	**2.00**	**2.00**	**2.00**	4.00 (1.50)	3.00 (2.25)	**2.00** (1.50)
Leptospermum scoparium (manuka)	3.00	**2.00**	**2.00**	**2.00** (0.83)	**1.00** (0.75)	**2.00** (1.50)
Origanum majorana (marjoram)	**2.00**	**2.00**	**0.50**	**2.00** (0.83)	4.00 (3.00)	**1.00** (1.25)
Cymbopogon martini (palmarosa)	4.00	**2.00**	**2.00**	4.00 (1.50)	**2.00** (1.50)	4.00 (3.00)
Mentha piperita (peppermint)	4.00	3.00	**1.00**	**2.00** (0.75)	**2.00** (1.33)	**2.00** (2.00)
Rosmarinus officinalis (rosemary)	**2.00**	**1.00**	**2.00**	**2.00** (1.00)	**2.00** (1.50)	**2.00** (1.50)
Melaleuca alternifolia (tea tree)	**2.00**	**1.00**	**2.00**	2.00 (1.00)	3.00 (3.00)	2.00 (1.00)
Thymus vulgaris (thyme)	**1.00**	**1.00**	**0.50**	**1.00** (0.75)	**1.00** (1.50)	**1.00** (1.00)
Ciprofloxacin (for bacteria) and Amphotericin B (yeast)	0.63×10^{-3}	0.31×10^{-3}	3.13×10^{-3}	0.63×10^{-3}	0.31×10^{-3}	$3.13\ 10^{-3}$

Bold = noteworthy antimicrobial activity; (values in brackets) = FICI; * = Combination value not calculated.

6.3. Overview of Chapter 6

- *Eucalyptus radiata* had comparable efficacy to other selected essential oils against *S. aureus*, *P. aeruginosa* and *C. albicans*.

- No obvious differences in susceptibility patterns were found between the Gram-positive *S. aureus* and the Gram-negative *P. aeruginosa*.

- When comparing the antimicrobial activity of all the selected essential oils, *T. vulgaris* demonstrated the best overall antimicrobial activity against all three pathogens, with average MIC's of 1.00 mg/ml, 1.00 mg/ml and 0.50 mg/ml against *S. aureus*, *P. aeruginosa* and *C. albicans* respectively.

- The most additive interactions were frequently demonstrated in the 1:1 *E. radiata* essential oil combinations against *S. aureus* (66.67%) in comparison to *P. aeruginosa* (8.33%) and *C. albicans* (16.67%).

- No undesirable antagonistic interactions were noted for any of the essential oils tested 12 combinations.

CHAPTER 7

The antiquorum sensing activity of *E. radiata* leaf essential oils independently and in combination with other selected essential oils

7.1. Introduction

There has been an increasing interest in the role of plant products such as essential oils in regulating interactions between micro-organisms (Koh *et al.*, 2013). However, the various modes of action by which *E. radiata* leaf essential oil exerts is effects on pathogenic micro-organisms are yet to be elucidated. This Chapter will give an overview of the inhibition of quorum sensing as a possible mode of action of *E. radiata* leaf essential oil.

In addition, the antiquorum sensing properties of *E. radiata* leaf essential oil will be screened at 1:1 combination with essential oils of: *C. bergamia* (bergamot), *L. angustifolia* (lavender), *C. limon* (lime), *O. majorana* (marjoram), *C. martini* (palmarosa), *M. piperita* (peppermint), *R. officinalis* (rosemary), *M. alternifolia* (tea tree), *T. vulgaris* (thyme), *Citrus aurantifolia* (lemon), *E. caryophyllus* (clove) and *L. scoparium* (manuka). These essential oils were selected for evaluation in this study based on their combination use with *E. radiata* (Chapter 1, Table 1.3) and their previously reported anti-QS activity (Khan *et al.*, 2009; Szabó *et al.*, 2010; Alvarez *et al.*, 2012; Jaramillo-Colorado *et al.*, 2012; Eris and Ulusoy, 2013; Kerekes *et al.*, 2013; Ahmad *et al.*, 2014b; Bai and Vittal, 2014; Luís *et al*., 2015). This study will be the first to screen the antiquorum sensing properties of *E. radiata* essential oil in combination with other oils. Screening of antiquorum sensing activity essential oils in combination will enable the evaluation of whether *E. radiata* oil may increase the anti-QS efficacy of these essential oils by taking advantage of possible synergistic interactions.

7.2. Results and discussion

7.2.1. Antiquorum sensing activity of essential oils

The percentage violacein inhibition of *C. violaceium* (ATCC 12472) pigment (violacein) production and the minimum quorum sensing inhibitory concentration's (MQSIC) of the essential oils are presented in Table 7.1. Anti-QS activity was observed for 92.30% of the screened essential oils (Table 7.1). In this study, *E. radiata*, *C. bergamia* (bergamot), *L. angustifolia* (lavender), *C. limon* (lime), *O. majorana* (marjoram), *C. martinii* (palmarosa), *M. piperita* (peppermint), *R. officinalis* (rosemary), *T. vulgaris* (thyme), *C. aurantifolia* (lemon), *E. caryophyllus* (clove) and *L. scoparium* (manuka) displayed anti-QS activities. The percentage violacein inhibition ranged 79.11% - 95.30% with MQSIC values of 0.06 - 2.00 mg/ml. The percentage inhibition displayed by the essential oils was higher than that of the positive control (vanillin) at 78.90% (Table 7.1).

Eucalyptus radiata at 0.50 mg/ml MQSIC inhibited violacein production by of 95.30%, had the highest overall percentage violacein inhibition in comparison to all other essential oils and the positive control vanillin (Table 7.1). This high percentage violacein inhibition value (95.3%) indicates that *E. radiata* oil is the oil with properties closest to total/complete (100%) inhibition of QS activity among all the tested essential oils. In corroboration with this study, Luís *et al.* (2015) also identified *E. radiata* as an 'efficient' inhibitor of violacein production, showing higher antiquorum sensing activity in comparison to the popular *E. globulus* essential oil.

All MQSIC values were observed at sub-MIC values as previously noted by Khan *et al.* (2009) and Alvarez *et al.* (2012), Table 7.1. The anti-QS activities of the essential oils were concentration dependent. The ability to inhibit violacein production decreased with decreasing concentration (Figure 7.1). For example, *L. scoparium* (manuka) displayed antiquorum sensing activity at 1.00 mg/ml, but at a lower concentration of 0.25 mg/ml, the essential oil potentiated quorum sensing activity (increased violacein production), Figure 7.1.

Although *E. radiata* has the highest percentage violacein inhibition, of the 13 essential oils screened, *T. vulgaris* (thyme) and *E. caryophyllus* (clove) showed the most noteworthy inhibition of violacein production with the a combination of lowest MQSIC values and high percentage inhibition of 0.06 mg/ml at 85.8% and 0.13 mg/ml at 91.7% respectively (Table 7.1). The most potent activity (lowest MQSIC values) at > 50.00% inhibition was displayed by *E. caryophyllus* (clove) and *T. vulgaris* (thyme) essential oil (Table 7.1). Similarly, Khan *et al.* (2009) and Eris and Ulusoy (2013) noted *E. caryophyllus* (clove) to exhibit significant anti-QS activity. *Thymus vulgaris* (thyme) exhibited noteworthy anti-QS activity in this study, however, no anti-QS activity was reported by Khan *et al.*, 2009, while weak activity was reported by Eris and Ulusoy (2013) for *T. vulgaris* (thyme).

Other essential oils previously reported to exhibit no anti-QS activity against *C. violaeum* ATCC 12472 were *C. bergamia* (bergamot), *C. martinii* (palmarosa), *R. officinalis* (rosemary), *C. aurantifolia* (lemon), upon evaluation using the disc diffusion assay (Khan *et al.*, 2009; Eris and Ulusoy, 2013). This difference in results may be due to the difference in methods used. Due to the volatile nature of essential oils, limitations of the disc diffusion method may lead to an underestimation of activity (van Vuuren, 2008). Szabó *et al.* (2010) reported *L. angustifolia* (lavender) and *R. officinalis* (rosemary) oils as potent QS inhibitors. These oils were also found to exhibit anti-QS activity in this study.

In this study, *M. alternifolia* (tea tree) oil was the only oil to show no antiquorum sensing activity when tested independently, while Alvarez *et al.* (2012) noted a > 80% violacein inhibition by a *M. alternifolia* (tea tree) sample at 0.50 µl/ml. Differences in properties of essential oils can often be attributed to variations in chemical composition. Unfortunately the chemical composition of the *M. alternifolia* (tea tree) oil sample screened by Alvarez *et al.* (2012) was not given. This would have served as a basis of comparison of the antiquorum sensing properties of the essential oils.

Thymus vulgaris (thyme) and *C. ymbopogon martinii* (palmarosa) were the only two oils that exhibited inhibition of violacein production at more than one concentration. These concentrations were 0.13 mg/ml and 0.06 mg/ml (*T. vulgaris*) and *C. martinii* 4.00 mg/ml and 2 mg/ml (Table 7.1). This is interesting to note as thyme (MIC 0.25 mg/ml) (MIC 8.00

mg/ml) represent the most and least active essential oils of the test samples. Although *C. martinii* (palmarosa) essential oil displayed the weakest/poor antimicrobial activity against *C. violacium* (ATCC 12472), the oil still possessed anti-QS activity at 2 mg/ml (90.0%). This finding goes to show that even though some essential oils have poor minimum inhibitory concentration values, these oils should not be over-looked as they may have anti-QS potential (Ahmad *et al.*, 2014b).

7.2.2. Antiquorum sensing activity of essential oils in 1:1 combinations with *E. radiata* leaf essential oil

Some studies are available describing the anti-QS activity of essential oils (Khan *et al.,* 2009; Szabó *et al.,* 2010; Jaramillo-Colorado *et al.,* 2012). However, this study is the first to report on the antiquorum sensing activity of essential oils in combination, keeping that essential oils are often used in combinations in the hope of enhancing activity (van Vuuren and Viljoen, 2011). Results of the interactions of the essential oil combinations were recorded as an FQSICI value, Table 7.1. When the 12 essential oils were placed in combination with *E. radiata* five of the combinations were additive (0.75 - 1.00 FQSICI), five non-interactive (1.25 - 1.50 FQSICI), and no FQSIC could be determined for combinations with *M. alternifolia* (tea tree) and *T. vulgaris* (thyme).

Thymus vulgaris (thyme) and *E. radiata* essential oils displayed anti-QS activity independently, however when combined at a 1:1 ratio the combination showed no anti-QS properties. The absence of antiquorum sensing activity of the *E. radiata*: *T. vulgaris* (thyme) combination could be due to 'antagonistic' interactions between essential oil compounds resulting in neutralised antiquorum sensing activity. In contrast, *M.a alternifolia* (tea tree) essential oil showed no anti-QS activity when tested independently, however, upon combination with *E. radiata* leaf essential oil at a 1:1 ratio, anti-QS activity was observed. The following hypothesis is presented: The antiquorum sensing activity of the *E. radiata*: *M.a alternifolia* (tea tree) could be due to additive or synergistic interactions between essential oil compounds, or could be as results of the *E. radiata* leaf essential. Therefore, to test this hypothesis, the major compounds of these two essential oils will be evaluated in the next

Chapter (Chapter 8) to investigate possible synergistic interactions between compounds that may be responsible for this observation.

Table 7.1. Antiquorum sensing activity of essential oils independently and in 1:1 combination with *E. radiata* leaf essential oil against *C. violaceum* ATCC 12472.

Independent oils				**1:1 combination with *E. radiata essential oil***			
Essential oil	**MIC (mg/ml)**	**MQSIC (mg/ml)**	**% Violacein inhibition**	**MIC 1:1 (mg/ml)**	**MQSIC 1:1 (mg/ml)**	**% Violacein inhibition**	**FQSICI**
Eucalyptus radiata	1.00	0.50	95.30	*	*	*	*
Citrus bergamia (bergamot)	4.00	2.00	89.30	2.00	1.00	87.40	1.25
Eugenia caryophyllus (clove)	0.25	0.13	79.80	0.50	0.25	90.30	1.25
Lavendula angustifolia (lavender)	2.00	1.00	89.00	1.00	0.50	84.30	0.75
Citrus aurantifolia (lemon)	2.00	1.00	89.00	1.00	0.50	86.30	0.75
Citrus limon (lime)	1.00	0.50	89.10	1.00	0.50	87.00	1.00
Leptospermum scoparium (manuka)	2.00	1.00	90.20	2.00	0.50	82.30	0.75
Origanum majorana (marjoram)	0.50	0.25	86.90	1.00	0.50	91.80	1.50
Cymbopogon martinii (palmarosa)	8.00	2.00	90.00	2.00	1.00	87.70	1.13
Mentha piperita (peppermint)	4.00	2.00	89.70	2.00	1.00	87.20	1.25
Rosmarinus officinalis (rosemary)	1.00	0.50	79.11	1.00	0.50	79.60	1.00
Melaleuca alternifolia (tea tree)	**0.50**	**no MQSIC**	**no MQSIC**	**1.00**	**0.50**	**87.20**	**no FQSICI**
Thymus vulgaris (thyme)	**0.25**	**0.06**	**85.80**	**0.25**	**no MQSIC**	**no MQSIC**	**no FQSICI**
Vanillin (positive control)	1.56	0.39	78.89	*	*	*	*

Bold = (differences in antiquorum sensing activity between essential oils tested independently versus oils in 1:1 combination with *E. radiata* leaf essential oil. *=no value

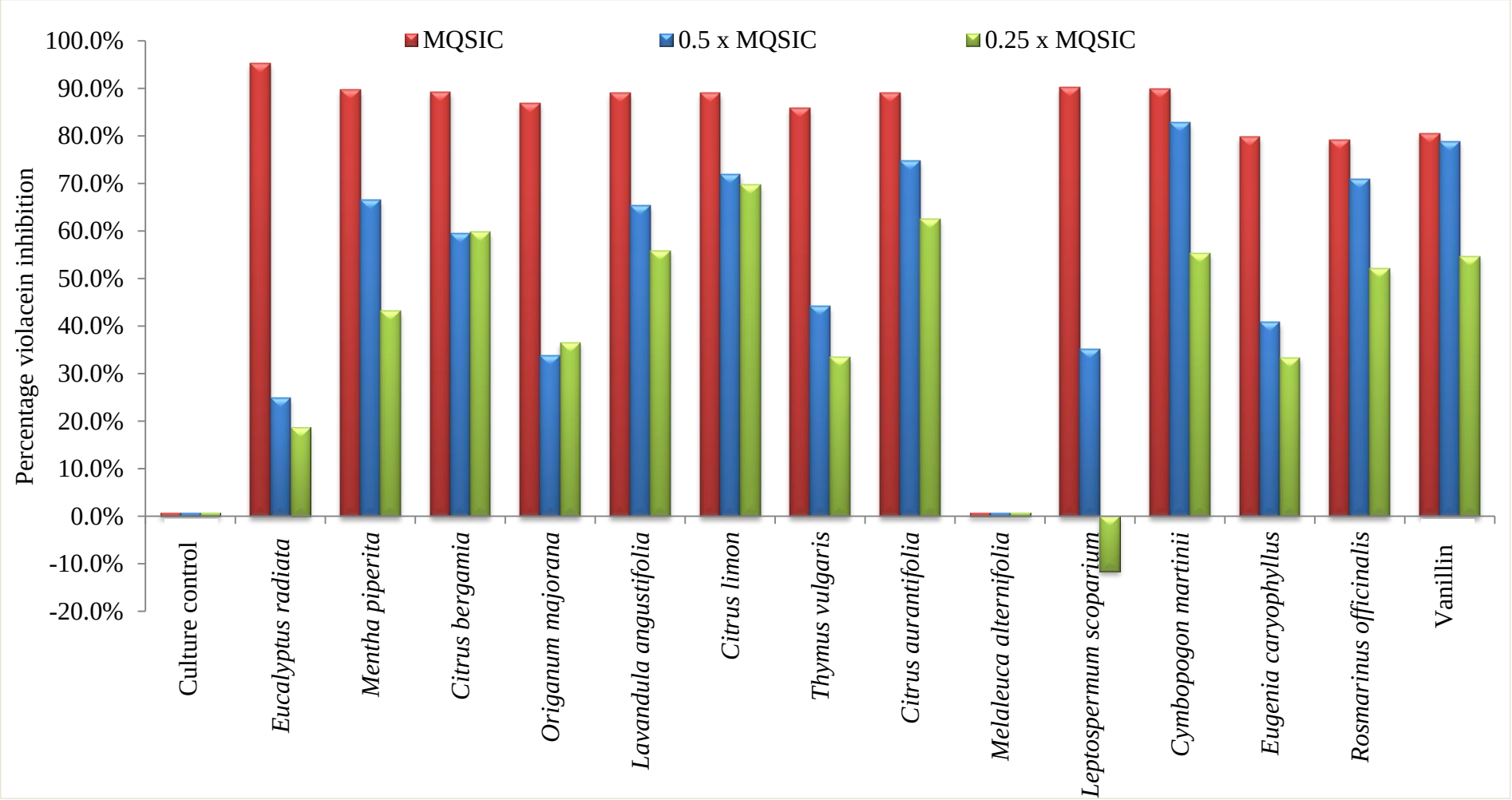

Figure 7.1. Quantitative analysis of *C. violaceum* ATCC 12472 violacein inhibition by selected essential oil compounds at MQSIC, 0.5xMQSIC and 0.25xMQSIC.

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7.3. Overview of Chapter 7

- *Eucalyptus radiata* displayed good antiquorum sensing activity against C. *violaceum* at 0.50 mg/ml MQSIC and 95.30% violacein inhibition.

- *Eucalyptus radiata* displayed the highest percentage violacein inhibition 95.30% in comparison to all other tested essential oils.

- *Eucalyptus radiata* displayed more efficient antiquorum sensing activity in comparison to all essential oils with the exception of *E. caryophyllus* (79.80% violacein inhibition at 0.13 mg/ml MQSIC and *T. vulgaris* (85.80% violacein inhibition at 0.06 mg/ml MQSIC.

- The 1:1 combination of *E. radiata: T. vulgaris* resulted in no antiquorum sensing activity.

- The 1:1 combination of *E. radiata: Me. alternifolia* resulted was the most promising combination, in which the combination resulted in an additive outcome.

CHAPTER 8

The role of major compounds on the anti-quorum sensing properties of *E. radiata* leaf essential oil combinations

8.1. Introduction

This chapter evaluates the antiquorum sensing properties of the major compounds of the *E. radiata* leaf essential oil against the biosensensor strain, *C. violaceum* ATCC 12472. These major compounds will be evaluated independently and in various combinations. Independent studies will allow the identification of active QS compounds within the *E. radiata* leaf essential oil. Combination studies will allow evaluation of possible synergistic interactions between the major compounds.

When essential oils are combined, the number of compounds within the essential oil mixture increases. Each essential oil contributes its compounds with unique properties. These compounds will interact with each other, resulting in an infinite number of interactions. The sum of which is the property of the mixture. Therefore, evaluating compound interactions can help with understanding the properties of an essential oil mixture.

The promising antiquorum sensing activity between *E. radiata* and *M. alternifolia,* suggest possible synergy between the two essential oils. To explore this further, the antiquorum sensing activity of the major compounds of *E. radiata* essential oil is evaluated in combination with the major compounds of *M. alternifolia* essential oil. To the best of our knowledge, there is no study on the possible interactions between individual molecules of the *E. radiata* leaf essential oil.

8.2. Results

8.2.1. The role of major compounds on the anti-quorum sensing properties of *Eucalyptus radiata* leaf essential oil

The MQSIC values and percentage violacein inhibition of all the compounds have been listed in Table 8.1. The major compounds of the *E. radiata* leaf essential oil evaluated are; 1,8-cineole and α-terpineol. 1,8-Cineole inhibited violacein production by 80.60% at 1.00 mg/ml MQSIC while α-terpineol inhibited violacein production by 82.60% at 2.00 mg/ml. Similarly, Ahmad *et al.*, (2014b) also reported the quorum sensing inhibitory concentration of α-terpineol (> 90.0% violacein inhibition at 0.13 mg/ml MQSIC).

To determine the possible combinational interactions of 1,8-cineole and α-terpineol, the fractional quorum sensing inhibitory concentration (FQSIC) was calculated. At a 1:1 ratio, the combination of 1,8-cineole and α-terpineol showed a synergistic interaction (0.19 FQSIC) with a 82.4% violacein inhibition. However, when combined at the relative ratio they occur in the essential oil, the potency of the combination to inhibit violacein production was significantly lowered to 32.70%. The differences in anti-quorum sensing activity of the compounds at 1:1 and relative ratios shows the variation that can occur when mixed at various combinations. This observation is suggestive of the involvement of minor compounds in the anti-quorum sensing activity of this essential oil. These minor compounds include: (+)-α-pinene, (-)-α-pinene, β-pinene and (+)-limonene, which have previously been reported to increase violacein production, while geraniol, linalool, *p*-cymene inhibited violacein production (Ahmad *et al.*, 2014b). Essentially, *E. radiata* oil is a mixture of compounds with the ability to either inhibit or promote anti-QS activity, therefore, the resultant anti-QS activity may be a result of the sum of the interactions of the compounds within the essential oil. In corroboration with the results of this study, Kerekes *et al.* (2013) also found that whole essential oils were better quorum sensing inhibitors in comparison to their major compounds.

8.2.2. The role of major compounds on the anti-quorum sensing properties of *M. alternifolia* essential oil

The major compounds of *M. alternifolia* essential oil were tested independently and in combination to investigate the anti-quorum sensing properties of the oil observed in the previous Chapter. Compounds comprising 10.00% of the essential oil composition were selected for screening. The major compounds of the *M. alternifolia* essential oil evaluated were: (+)-terpinene-4-ol, (-)-terpinene-4-ol, α-terpinene and γ-terpinene. All of the major compounds of *M. alternifolia* exhibited anti-quorum sensing activity (Table 8.1). The highest anti-quorum sensing activity was noted for (+)-terpinene-4-ol which inhibited violacein production by 91.4% at 0.25 mg/ml MQSIC and (+)-terpinene-4-ol inhibited violacein production by 92.4% at 0.25 mg/ml MQSIC. Overall, both enantiomers of terpinene-4-ol ((+)-terpinene-4-ol and (-)-terpinene-4-ol) displayed better anti-quorum sensing activity in comparison to the other compounds. Ahmad *et al.*, (2014b), noted a difference in anti-quorum sensing activity between (+) and (-)-enantiomers of other compounds (e.g. limonene, and carvone), therefore the effect of stereochemistry was also evaluated in this study to evaluate the effect of stereochemistry. In contrast, the results (Table 8.1) showed no significant difference in stereochemistry between the (+) and (-)-enantiomers of terpinene-4-ol.

At a 1:1 ratio, only indifferent interactions were observed with FQSIC values of 1.25 with 81.70 - 82.80% violacein inhibition (Table 8.1). In contrast, antagonism was observed when compounds were combined at the relative ratios they occur within the essential oil. No anti-quorum sensing activity was noted for 83.33% of the combinations. The remaining 16.67% of the combinations resulted significantly lowered violacein inhibition (32.70%), this was the combination of α-terpinene and γ-terpinene.

Interestingly, *M. alternifolia* oil showed no anti-quorum sensing properties when tested independently (Chapter 7, Table 7.1). However, *M. alternifolia* leaf essential oil is a source of compounds with anti-quorum sensing activity. Terpinene-4-ol ((+)-terpinene-4-ol and (-)-terpinene-4-ol) α-terpinene and γ-terpinene demonstrated anti-quorum sensing properties (Table 8.1). This shows that the presence of compounds with quorum sensing inhibitory properties do not necessarily guarantee anti-quorum sensing properties within the whole

essential oil, regardless of the ratio of those constituents within the essential oil. With *M. alternifolia*, the significance of compound ratios was pronounced.

8.2.3. Antiquorum sensing activity of *E. radiata* and *M. alternifolia* major compounds in combination

In comparison to 1:1 combinations, the general trend observed when the compounds from both oils were combined at relative ratios was a decrease in anti-quorum sensing activity. At a 1:1 combination ratio the violacein production ranged between 74.00 - 82.80%, while at various ratios the violacein production ranged from no MQSIC to 34.6%. Compounds combined at a 1:1 ratio produced FQSICI values ranging from 0.19 - 1.25 (Table 8.1) corresponding to synergistic additive or indifferent interactions. Two combinations displayed synergistic interactions, these were; α-terpinene and α-terpineol (ΣFQSIC 0.19), and 1,8-cineole and α-terpineol (FQSICI 0.19) at 1:1 ratios. Interestingly, both synergistic combinations contained α-terpineol. α-Terpineol was previously reported to exhibit > 90.00% inhibition at 0.13 mg/ml (Ahmad *et al.*, 2014b). This suggests that the synergy in the combinations might be due to the presence of α-terpineol.

The differences in anti-quorum sensing properties points to the significance of compound ratios in the resultant properties of the essential oil blend. This impact of compound ratios was also previously noted by van Vuuren and Viljoen (2007), reporting how the type of interactions observed depended on the ratio of the compounds.

The *E. radiata* and *M. alternifolia* essential oil combination (87.20% violacein inhibition at 0.50 MQSIC) exhibited anti-quorum sensing activity regardless of the lack of anti-quorum sensing activity exhibited by its compounds at the relative ratios they appear in the mixture (Table 8.1). In addition the essential oil mixture, inhibited violacein production at 87.2%, which is much higher than any of the major compounds at relative ratios. Therefore, the higher antiquorum sensing properties of the essential combination may be due to the involvement of minor compounds. These minor compounds may be interacting with these major compounds in a synergistic manner, the sum of which results in the good anti-QS

properties of the combination of the whole oil. Further evaluation of interactions between minor and major compounds is required in order to assess this hypothesis.

Majority of the no MQSIC (no anti-QS activity/no violacein inhibition) outcome resulted from the combinations of *M. alternifolia* (tea tree) compounds at various ratios they naturally occurred within the essential oil (Table 8.1). This observation may explain why *M. alternifolia* (tea tree) had no anti-quorum sensing activity even though its major compounds exhibited anti-quorum sensing activity independently.

8.2.4. Mode of action

The QS system of the *C. violaceium* (ATCC 12472) biosensor strain used in this study consists of a CviI (auto-inducer synthase homologue)/CviR (cognate receptor homologue) circuit. This CviI/CviR circuit controls virulence (Stauff and Bassler, 2011). *Chr. violaceium* (ATCC 12472) produces and responds to cognate auto-inducer molecules, N-hexanoyl-L-acylhomoserine lactone (C6-AHL) and C4-AHL. These acylated homoserine molecules bind to the CviR receptor. The result of this complex is the induction of the expression of violacein production (Stauff and Bassler, 2011; Alvarez *et al.*, 2012; Chenia, 2013; Koh *et al.*, 2013). A deduction is made from the results that *E. radiata* leaf essential oil has the ability to either inhibit cognate auto-inducer molecules C6-AHL and C4-AHL, inhibition of signal molecule synthesis, break down of AHL molecules or block AHL receptors. Results from this study confirms that *E. radiata* leaf essential oil indeed comprises of virulence modifying compounds within its essential oil, confirming its potential for use as a source of anti-QS compounds.

Table 8.1 Mean MQSIC of *E. radiata* and *M. alternifolia* (tea tree) oil major compounds independently and in combination with ΣFQSIC, determined for 1:1 combinations at various ratios against *C. violaceum* ATCC 12472.

Quorum sensing inhibitor	**MQSIC (mg/ml)**	**% Violacein inhibition**
Independent		
(+)-Terpinene-4-ol (*M. alternifolia*)	0.25	91.40
(-)-Terpinene-4-ol (*M. alternifolia*)	0.25	92.40
α-Terpinene (*M. alternifolia*)	1.00	83.30

Quorum sensing inhibitor	MQSIC (mg/ml)	% Violacein inhibition
y-Terpinene(*M. alternifolia*)	2.00	33.70
α-Terpineol (*E. radiata*)	2.00	82.60
1,8-Cineole (*E. radiata*)	1.00	80.60
1:1 Combinations		
1,8-Cineole:(-)-Terpinene-4-ol	0.50 (1.25)	80.60
1,8-Cineole:(+)-Terpinene-4-ol	0.50(1.25)	82.40
1,8-Cineole : α-Terpineol	0.25 (**0.19)**	82.40
1,8-Cineole:α-Terpinene	1.00 (1.00)	74.00
(-)-Terpinene-4-ol:α-Terpineol	0.50 (1.13)	82.80
(+)-Terpinene-4-ol:α-Terpineol	0.50 (1.13)	81.90
(-)-Terpinene-4-ol: α-Terpinene	0.50 (1.25)	81.70
(+)-Terpinene-4-ol: α-Terpinene	0.50 (1.25)	82.80
α-Terpinene:α-Terpineol	0.25 (**0.19**)	81.70
Various ratios (relative to essential oil composition within the 1:1 essential oil combination)		
1,8-Cineole: α-Terpinene	*	32.70
α-Terpinene:(-)-Terpinene-4-ol	*	no MQSIC
α-Terpinene:(+)-Terpinene-4-ol	*	no MQSIC
α-Terpinene:α-Terpineol	*	no MQSIC
α-Terpinene:y-Terpinene	*	33.70
y-Terpinene:1,8-cineole	*	32.70
y-Terpinene:(-)-Terpinene-4-ol	*	no MQSIC
y-Terpinene:(+)-Terpinene-4-ol	*	no MQSIC
y-Terpinene:α-Terpineol	*	no MQSIC
(-)-Terpinene-4-ol: 1,8-Cineole	*	33.70
(+)-Terpinene-4-ol: 1,8-Cineole	*	34.60
(-)-Terpinene-4-ol: α-Terpineol	*	no MQSIC
(+)-Terpinene-4-ol: α-Terpineol	*	no MQSIC
1,8-Cineole:α-Terpineol	*	33.70
Vanillin	0.39	78.90

Bold = Synergistic interaction; * = No value calculated.

8.3. Overview of Chapter 8

- Major compounds in *E. radiata* essential oil possess anti-quorum sensing activity. 1,8-Cineole displayed 80.60% violacein inhibition at 1.00 MQSIC. α-Terpineol displayed 82.60% violacein inhibition at 2.00 MQSIC.

- Two synergistic interactions were noted with the 1:1 combinations of α-terpinene and α-terpineol (ΣFQSIC 0.19), and 1,8-cineole and α-terpineol (FQSICI 0.19).

- Loss of anti-quorum sensing activity was noted only at relative combination ratios.

Chapter 9
Dissertation summary, conclusions and future recommendations

9.1. Dissertation summary

The effects of the seasonal variation and leaf age on the yield, chemical composition and resultant antimicrobial activity of *E. radiata* leaf essential oil was evaluated. The essential oil was also screened for anti-quorum sensing activity in order to evaluate its anti-pathogenic potential. *Eucalyptus radiata* essential oil, was investigated in combination with 12 other essential oils in order to establish some antimicrobial evidence for its use in combination. Finally, major compounds were evaluated independently and in combination in order to investigate their correlation to the antimicrobial and anti-quorum sensing properties of the *E. radiata* leaf essential. The major findings of the study aligned against the objectives are listed below:

9.1.1. Objective 1: Effects of seasonal variation on the yield and chemical composition of young and mature *E. radiata* leaf essential oil (Chapter 3)

The essential oil yields of the 23 *E. radiata* leaf essential oil samples ranged from 0.14% to 4.31% due to seasonal variation. The highest essential oil yields can be obtained during spring/summer months (high rainfall and high temperature conditions) for both young and mature leaf oil samples. Essential oil was obtained from both young and mature leaves, however, mature leaves were identified to produce higher yields. A total of 26 compounds were identified and the major compounds were 1,8-cineole (65.7% ± 9.5), α-terpineol (12.8% ± 4.4) and limonene (6.5% ± 2.4). This was expected, as 1,8-cineole is one of the major compounds in other medicinal type *E. radiata* essential oils (Pearson, 1993; Sing, 1994; Chisowa, 1997; da Cruz Francisco *et al.*, 2001; Bendaoud *et al.*, 2009; Guba, 2009; Juan *et*

al., 2011; Mulyaningsih *et al.*, 2011). Seasonal variation and leaf age resulted in quantitative variations in essential oil composition of the 23 *E. radiata* leaf essential oil samples.

Analysis of the essential oil yield and chemical composition has shown that there is great potential for the use of the Tzaneen harvested *E. radiata* species as medicinal *Eucalyptus* oil source. The essential oil profile is similar in terms of 1,8-cineole content when compared to its commercial counterpart *Eucalyptus radiata*-comm (65.4% 1,8-cineole content).

9.1.2. Objective 2: Antimicrobial activity of *E. radiata* leaf essential oil (Chapter 4)

Eucalyptus radiata leaf essential oil displayed noteworthy antimicrobial activity against a broad-spectrum of pathogens associated with dental, gastrointestinal/food-related, wound/skin and respiratory infections. The quantitative variations in chemical composition as a result of seasonal variation had no significant impact on the antimicrobial activity observed. Moreover, variation in antimicrobial activity was not dependent on whether young or mature leaf oil was used, but rather on the pathogen studied. In general, the streptococci and *L. acidophilus* were the most susceptible pathogens to the inhibitory effects of *E. radiata* leaf essential oil, in particular, the dental pathogens *S. mutans* (0.25 - 1.00 mg/ml) and *L. acidophilus* (0.19 - 1.75 mg/ml). The good antimicrobial activity observed against *S. pneumoniae, S. agalactiae* and *S. pyogenes* justify its use for respiratory conditions (Rose and Earle, 1996; Mulyaningsih *et al.*, 2011). The broad-spectrum activity might provide some rationale for the use of *E. radiata* essential oil in the treatment of a variety of infectious conditions in aromatherapy (Rose and Earle, 1996; Balz, 1999; Synovitz and Larson, 2012).

Prior to this study, no research had been conducted on the antimicrobial activity of *E. radiata* against *S. sonnei*, *L. acidophilus* and *B. cereus*.

The commercial potential for the use of *E. radiata* leaf essential oil as a source of commercial medicinal oil holds enormous promise due to favourable yields, a conservative chemical profile showing negligible variation in the antimicrobial activity of young and mature leaves. Moreover, the essential oil showed similar antimicrobial activity to its commercial counterpart *E. radiata*-comm, the popular *E. globulus* and the primary source of medicinal

Eucalyptus oil in South Africa, *E. smithii*. In addition, this plant is cultivated by a resident farmer (Mr Bruce Stumbles), which means it is easily accessible to the South Africa essential oil market. Findings from this study may serve as *in vitro* rationale for its current applications in infectious conditions.

9.1.3. Objective 3: The role of major compounds on the antimicrobial properties of *E. radiata* leaf essential oil (Chapter 5)

The major compounds showed noteworthy antimicrobial activity against all five pathogens (*S. pyogenes*, *S. pneumoniae*, *S. agalactiae*, *L. acidophilus* and *S mutans*). Of the three compounds (S-(-)-limonene and R-(+)-limonene) at 0.25 - 0.75 mg/ml showed the highest antimicrobial activity, followed by α-terpineol (0.75 - 1.00 mg/ml) and the lowest activity was noted for 1,8-cineole (2.00 mg/ml). All 16 synergistic interactions from 1:1 and various ratios resulted from combinations with limonene. Limonene was identified to have the strongest influence on strength of the antimicrobial activity of the combinations.

In comparison, to the crude essential oil displayed relatively higher antimicrobial activity in comparison to its major compounds against *S. pneumoniae*, *S. agalactiae*. This observation points to the possible contributory role of other compounds (minor compounds) towards the antimicrobial activity of the essential oil. The combination of major compounds assayed in 1:1 blends and various ratios demonstrated the significance of compound ratios on the interactions and antimicrobial properties.

9.1.4. Objective 4: The antimicrobial properties of *E. radiata* in 1:1 combinations with selected essential oils (Chapter 6)

When *E. radiata* was mixed in 1:1 ratios with various essential oils, only additive and indifferent interactions were observed. The most additive interactions were frequently demonstrated for the 1:1 *E. radiata* essential oil combinations against *S. aureus* (66.67%) in comparison to *P. aeruginosa* (8.33%) and *C. albicans* (16.67%). None of the 1:1 combinations resulted in noteworthy (synergistic) or undesirable (antagonistic) (synergy or antagonism) interactions.

9.1.5. Objective 5: The anti-quorum sensing activity of *E. radiata* leaf essential independently and in combination with other selected essential oils (Chapter 7)

Eucalyptus radiata at 0.50 mg/ml MQSIC inhibited violacein production by 95.3%, confirming that *E. radiata* essential oil has antiquorum sensing properties. Moreover, this indicates that *E. radiata* oils is a source of active anti-QS compounds. Other essential oils exhibiting noteworthy anti-quorum sensing activity were *T. vulgaris* (0.06 mg/ml MQSIC at 85.80%) and *E. caryophyllus* (0.13 mg/ml MQSIC at 79.8%) and *O. marjorana* (0.25 mg/ml MQSIC at 86.9%) which all showed better anti-quorum sensing activity in comparison to the positive control, vanillin (0.39 mg/ml MQSIC at 78.9%).

When tested in 1:1 ratios, 41.6% of the combinations were additive while 41.6% non-interactive. The remaining 16.8% were representative of no FQSICI values (Table 7.1). Independently, *M. alternifolia* (tea tree) essential oil showed no antiquorum sensing properties. However, anti-quorum sensing activity was observed when evaluated in a 1:1 combination with the *E. radiata* essential oil. The 1:1 combination of *T. vulgaris* (thyme) and *E. radiata* essential oils showed no anti-quorum sensing properties, whereas independently the essential oils exhibited anti-quorum sensing properties.

9.1.6. Objective 6: The role of major compounds on the antiquorum sensing properties of *E. radiata* leaf essential oil combinations (Chapter 8)

1,8-Cineole inhibited violacein production by 80.6% at 1.00 mg/ml, while α-terpineol inhibited violacein production by 82.6% at 2.00 mg/ml. This confirms *E. radiata* leaf essential oil as a source of compounds with anti-QS activity. Only two synergistic interactions were observed with the 1:1 combinations of 1,8-cineole and α-terpineol (FQSICI 0.19) and α-terpineol and α: terpinene (FQSICI 0.19). Anti-quorum sensing activity can be achieved with combination

9.3. Conclusion

This comprehensive analysis of *E. radiata* essential oil has demonstrated for the first time, that this oil sourced from South Africa has a chemical consistency over a 12 month testing period. The oil has a favourable profile, (65.70% ± 9.50 1,8-cineole) and has antimicrobial potential that rivals other commercial *Eucalyptus* species, including the popular *E. globulus*. Furthermore, *E radiata* essential oil has been verified for antiquorum sensing properties, which serves as an alternative mode of action to conventional antimicrobials. The antimicrobial screening of *E. radiata* leaf essential oil in 1:1 combinations with commonly blended commercial essential ois showed additive and indifferent interactions and no antagonistic interactions, which reduces the concern with its combination use. This study showed that the underlying mechanisms of actions responsible for the antimicrobial and antiquorum sensing activities of the essential oil are not solely dependent on its major compounds (1,8-cineole;α-terpineol and limonene). Rather, its properties may be dependent on the sum of the interactions major and minor compounds at relative ratios within the essential oil, which requires further scientific evaluation.

9.2. Future recommendations

9.2.1. Toxicity studies

Like most complementary medicines, there has always been the misconception that essential oils are harmless by virtue of them being natural products (Little, 2004; Shishir, 2011). As stated in previous chapters, *E. radiata* is an important commercial medicinal oil source. It is often preferred over the popular *E. globulus* species by aromatherapists and referred to as the 'gentler' of the *Eucalyptus* essential oils (Rose and Earle, 1996; Mulyaningsih *et al*., 2011; Tourles, 2012; Luís *et al*., 2015). In spite of its popularity, very little is known about its toxicity and thus its safety. This perception that *E. radiata* essential oil is 'gentler' may be misinterpreted due to the ignorance of its potential toxicity. Therefore it is important for future research to perform cytotoxicity assays to demonstrate the oil is not toxic for the cells to minimise risk of harm in a therapeutic context.

The toxicity of *Eucalyptus* essential oil has been documented in literature, with side effects ranging from skin irritation to loss of consciousness (Balacs, 1997; Darben *et al.*, 1998; Shishir *et al.,* 2011; Mubarak *et al*., 2015). However, very little is known about the toxicity profiles of most specific species including *E. radiata*. This is because sometimes, it is often not specified in literature which *Eucalyptus* essential oil is being reported on, although it is always assumed to be *E. globulus* as it is the most popular species. For example, a patient presented with ulceration in the oral after topical application of *Eucalyptus* essential oil on their gums associated with a decaying tooth. This ulceration was a result of a chemical injury from the compounds within the essential oil, one particular suspected compound was 1,8-cineole (Shishir *et al.,* 2011). This case study presented by Shirshir *et al*. (2011), did not specify which *Eucalyptus* species was being referred to, nor was the composition of the essential oil reported. In this study, *E. radiata* essential oil showed great potential for application in dental conditions. In addition, the essential oil also contains high amounts of 1,8-cineole, which is implicated in the ulceration and also reported to have toxic effects on the nervous system and the gastrointestinal tract (Guba, 2009; Tisserand and Young, 2014). From this case report, it is evident that future research needs to include toxicity studies to determine the safety of *E. radiata* essential oil. In keeping with the theme of this study, future toxicity studies could be conducted across a one year period to evaluate how seasonal variation affects the toxicity profile of the essential oil.

9.2.2. Combinations studies of more than two essential oils

Essential oils are rarely used singularly. According to the literature review of this study, *E. radiata* essential oil is mostly found in preparations comprising more than two other essential oils (Chapter 1, Table 1.3). Therefore a more practical approach to future combination studies should involve the evaluation of more than two essential oils. Preferably more combination studies will be undertaken with the traditional and commercial herbal preparations, keeping in mind the relative ratios of the essential oils within the preparations and their therapeutic indications.

9.2.3. Evaluation of biofilm inhibition activity

The biofilm inhibition activities of other *Eucalyptus* essential oils such as *E. camaldulensis* and *E. globulus* have already been established (Rasooli *et al.*, 2009; Goldbeck *et al.*, 2014). In this study, *E. radiata* leaf essential oil showed similar antimicrobial efficacy to the essential oils of these *Eucalyptus* species. Therefore future studies should evaluate whether the oils also possesses anti-biofilm activity. The anti-quorum sensing activity displayed by the *E. radiata* leaf essential oil in this study highlights it potential application in anti-biofilm formation of *S. mutans*. The significance of this correlation is that *S. mutans* is associated with dental plaques. Dental plaques are biofilms, the formation of which is regulated by the quorum sensing system. The adhesion of this micro-organism is a crucial part of its pathogenicity. Attachments with biofilms make it hard to treat and remove, often requiring a combination of mechanical (for example, brushing with toothbrushes, flossing, oral irrigation) and chemical (for example, toothpaste, mouthwash) treatment approaches. The ability of *E. radiata* leaf essential oil to inhibit adhesion of *S. mutans* (Takarada *et al.*, 2004) combined with the anti-quorum sensing potential indicated in this study suggests that *E. radiata* oil is a good candidate as a source of phytoconstituents to help modify the development of biofilms by *S. mutans*. Biofilms, one of the products of quorum sensing, are also one of the leading causes of food spoilage and foodborne diseases (Kerekes *et al.*, 2013).

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APPENDIX A

Abstract for oral presentation at the South African Association of Botanists (SAAB) 41st annual conference

The potential for *Eucalyptus radiata* leaf essential oil use as a commercial antimicrobial

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Abstract

Due to their bioactive components, essential oils are gaining momentum as effective antimicrobial agents for food and pharmaceutical applications. The essential oil of *Eucalyptus radiata* is one of the few understudied commercially produced medicinal *Eucalyptus* essential oils, with a broad-spectrum of antiinfective therapeutic applications. Young and mature leaves of the *Eucalyptus radiata* species were sampled monthly, over a period of one year (January 2014 to December 2014); samples were evaluated for effects of seasonal variation on the essential oil yield, chemical composition and antimicrobial activity. Essential oils were obtained using the hydro-distillation method. Chemical composition was analysed by one and two dimensional gas chromatography coupled to mass spectrometry (GC-MS). The antimicrobial activity was analysed using the minimum inhibitory concentration (MIC) assay. The sum of the fractional inhibitory concentration (ΣFIC) was used to determine the types of interactions observed from the 1:1 combinations of *E. radiata* leaf essential oils with other essential oils. Essential oil yield was largely influenced by the phenological growth stage of

the leaf and seasonal variation. *Eucalyptus radiata* essential oil showed similar antimicrobial activity in comparison to essential oils from other *Eucalyptus* species. Independent 1:1 combinations showed non-interactive effects. *Eucalyptus radiata* essential oil showed broad-spectrum antimicrobial activity against the 17 selected test micro-organisms. *Pseudomonas aeruginosa* ATCC 27853, *Streptococcus mutans* NCTC 10919, *Lactobacillus acidophilus* ATCC 314, *Streptococcus agalactiae* ATCC 55618 and *Streptococcus pyogenes* NHLS 8668, showed the highest sensitivities (MIC values ≤ 1 mg/ml). The observed consistent noteworthy antimicrobial activity throughout the testing period (autumn, winter, spring, summer) provides credence to the reliable antiinfective properties of the *E. radiata* essential oil. Correlations from this study will contribute towards establishing a base of information to guide harvesting protocols towards the production of an ideal *E. radiata* essential oil quality and quantity as a commercial antimicrobial.

APPENDIX B

Abstract for oral presentation at the School of Therapeutic Sciences Biennial Research Day (University of the Witwatersrand)

Therapeutic potential for the use of *E. radiata* oil as an antimicrobial

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Abstract

Introduction

Eucalyptus radiata is one of the medicinal essential oil species used for a range of antiinfective conditions. Yet, *E. radiata* remains one of the most understudied medicinal *Eucalyptus* essential oils. In order for a better understanding of the therapeutic potential of this oil, research is required in order to establish a scientific basis for efficacy.

Objectives

To determine a rationale for its antiinfective uses through the evaluation of the antimicrobial activity of the essential oil against 18 pathogens selected based on the therapeutic applications of the essential oil. Antiquorum sensing activity will be evaluated in order to determine the anti-pathogenic potential of the *E. radiata* leaf essential oil. Another aspect will be to determine the chemical composition on an annual basis.

Methods

Essential oil was obtained from the hydrodistillation of young and mature leaf samples collected monthly over a period of one year (January 2014 to December 2014). The chemical composition of the essential oils was determined using one and two dimensional gas chromatography coupled to mass spectrometry (GC-MS). The antimicrobial activity was analyzed using the minimum inhibitory concentration assay minimum inhibitory concentration (MIC) assay. Antiquorum sensing activity was determined using the broth macrodilution assay against the biomonitor strain *Chromobacterium violaceium* ATCC 12742.

Results

The major compounds were 1,8-cineole (64.1% ± 11.9), α-terpineol (12.4% ± 4.6) and limonene (3.6% ± 2.7). *Eucalyptus radiata* leaf essential oil displayed noteworthy broad-spectrum antimicrobial activity against all 18 test pathogens. The highest susceptibility was noted against the Streptococci (0.19 mg/ml - 2.00 mg/ml) and *Lactobacillus acidophilus* (0.19 - 1.75 mg/ml). *Eucalyptus radiata* leaf essential oil displayed antiquorum sensing activity against *C. violaceium* 12472 with a 95.3% percentage violacein inhibition at a sub-MIC value of 0.50 mg/ml.

Conclusions

This study provides credence to the antiinfective properties attributed to the *E. radiata* essential oil. *Eucalyptus radiata* oil has shown the greatest potential for application in the management of dental conditions associated with *S. mutans* and *L. acidophilus*. The observed antiquorum sensing activity observed indicates the potential application of the *E. radiata* leaf essential oil as a preservative and management of microbial pathogenicity.

APPENDIX C

Abstract for publication submitted to the *Journal of Essential Oil Research*

Chemical composition and antimicrobial activity of South African *Eucalyptus radiata* leaf essential oil, sampled over a year

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ABSTRACT

This study investigated the seasonal variation of the chemical composition and antimicrobial activity of *Eucalyptus radiata* leaf essential oil. Young and mature *Eucalyptus radiata* leaf material was collected monthly (January 2014 to December 2014), hydrodistilled and analyzed using GC-MS. Essential oil yields ranged from 0.14% to 4.31% (w/w). The major compounds were 1,8-cineole (65.7% ± 9.5), α-terpineol (12.8% ± 4.4) and limonene (6.5% ± 2.4). Chemometric tools were used to determine seasonal variations, which showed slight variance in *E. radiata* chemistry between seasons. The minimum inhibitory concentration (MIC) assay showed that the highest activity was noted against the Streptococci (0.19-2.00 mg/mL) and *Lactobacillus acidophilus* (0.19-1.75 mg/mL). The activity of the *E. radiata* leaf essential oil is dependent on the unique ratio of its compounds. The *E. radiata* leaf essential oil showed good oil yields, a relatively consistent chemical profile and noteworthy antimicrobial activity that rivals other commercial Eucalypt counterparts.

KEYWORDS

Eucalyptus radiata; seasonal variation, antimicrobial activity; chemometric analysis; major compound.

APPENDIX D

Ethics clearance certificate for microbial cultures

Human Research Ethics Committee (Medical)

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Ref: W-CJ-140627-1 27/06/2014

TO WHOM IT MAY CONCERN:

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

Investigator: Prof S van Vuuren, Gillian D Mahumane (student no 363396)

Project title: Antimicrobial activity and chemical analysis of *Eucalyptus radiata* leaf essential oil.

Reason: The investigation is an *in vitro* laboratory study on stored cultures: *Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans, Bacillus cereus, Enterococcus faecalis, Listeria monocytogenes, Escherichia coli, Salmonella typhi, Shigella sonnei, Streptococcus pneumoniae, Streptococcus pyogenes, Klebsiella pneumoniae, Moraxella catarrhalis, Cryptococcus neoformans, Lactobacillus acidophilus, Streptococcus agalacticae and Streptococcus mutans.* No research on humans is included.

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Professor Peter Cleaton-Jones

Chair: Human Research Ethics Committee (Medical)

Copy - HREC(Medical) Secretariat: Anisa Keshav, Zanele Ndlovu.