The prediction of synergy in essential oil combinations used in the treatment of respiratory infections through experimental design and chemometric modeling

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Stephanie Leigh-de Rapper

A thesis submitted to the Faculty of Health Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree Doctor of Philosophy.

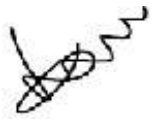
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Declaration: Student's contribution to article(s) and agreement of co-author(s)

I, Stephanie Leigh de Rapper, student number [0704317T], declare that this thesis is my own work and that I contributed amply towards research findings published in the article(s) stated below which are included in my thesis. This thesis is being submitted in fulfilment for the degree of Doctor of Philosophy in Pharmacy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

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Article 1

Title: Odoriferous therapy: A review identifying essential oils against pathogens of the respiratory tract

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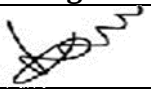
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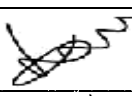
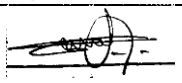
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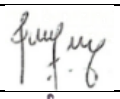
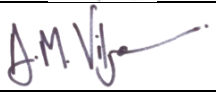
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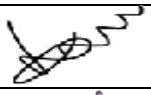
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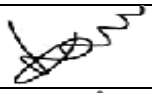
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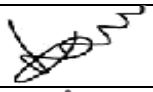
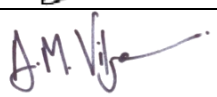
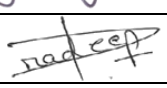
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Dedication

This dissertation is lovingly dedicated to my children, Ella Faith and Knox Evan de Rapper.

Your support, sacrifice, and constant love have sustained me; and will continue to do so throughout my life. May this work represent a symbol of dedication, and show you that you can do, and achieve any and all of your dreams.

I love you both.

List of presentations arising from this study

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2. Leigh-de Rapper, S., Viljoen, A.M., van Vuuren, S.F. *Making Connections: Linking redolent synergy to the aroma therapeutic treatment of respiratory tract infections (Poster)* 1st Annual Synergy Conference. University Club, Bonn, Germany, 9-10 March 2018.

3. Leigh-de Rapper, S., Viljoen, A.M., van Vuuren, S.F. *Making Connections: Linking redolent synergy to the aroma therapeutic treatment of respiratory tract infections (Poster)* Faculty of Health Sciences Research Day and Postgraduate Expo. The University of the Witwatersrand Research Day, Johannesburg, South Africa, 6 September 2018.

4. Leigh-de Rapper, S., Kamatou, G.P.P., Tankeu, S., Viljoen, A.M., van Vuuren, S.F. *Therapeutic blending: The use of essential oils in combination against pathogens of the respiratory tract (Podium Presentation)* 50th Annual International Symposium of Essential Oils (ISEO). University of Veterinary Medicine, Vienna, Austria, 9-11 September 2019.

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Abstract

The therapeutic use of essential oils, or aromatherapy, is a popular practice of complementary and alternate medicine for the treatment and management of respiratory infections. Essential oils are most often applied topically via massage or via inhalation. Fifty-five essential oils and 369 combinations were identified for the management of respiratory infections. As scientific evidence supporting these indications was lacking, this study intended to explore this avenue of research.

The minimum inhibitory concentration (MIC) was recorded for each identified essential oil against nine test organisms (*Staphylococcus aureus* ATCC 25924, *Streptococcus agalactiae* ATCC 55618, *Streptococcus pneumoniae* ATCC 49619, *Streptococcus* pyogenes ATCC 12344, *Haemophilus influenzae* ATCC 19418, *Klebsiella pneumoniae* ATCC 13883, *Moraxella catarrhalis* ATCC 23246, *Mycobacterium smegmatis* ATCC 19420 and *Cryptococcus neoformans* ATCC 14116). Gas chromatography coupled to mass spectrometry (GC-MS) was utilised to confirm purity of the essential oils and to identify the chemical profiles. The data from the GC-MS and antimicrobial studies was then analysed using chemometrics, a multivariate tool used to determine structure-activity relationships. Orthogonal projections to latent structure models were created and the compounds responsible for antimicrobial activity identified. The antimicrobial and toxic effects of the essential oils were then studied in 1:1 combinations using the MIC and brine shrimp lethality assay (BSLA), respectively. The interactive profiles of the combinations (antimicrobial and toxicity) were determined by calculating the fractional inhibitory concentration (ΣFIC) where either synergy, additivity, indifference or antagonism could be identified. Forty-nine essential oils and twenty-four essential oil combinations (1:1) were studied further for anti-inflammatory effects due to the favorable results of these oils in the MIC assay and BSLA. The anti-inflammatory activity was assessed by measuring the inhibition of nitric oxide (NO), an inflammatory mediator, production in LPS-activated RAW 264.7 macrophages. These oils were further investigated for potentially toxic effects using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) against human lung cell lines (A549). Based on the results of the anti-inflammatory and cell viability assessment against A549 cell lines, five essential oil combinations were found to be promising in equal combinations. These five combinations were then studied further by means of variable ratios using MIC analysis against the nine micro-

organisms associated with the respiratory tract. The variable ratios were plotted on isobolograms and then expanded where the MIC results were assessed using the synergy prediction software, SynergyFinder. The results of the isobolograms were compared to the SynergyFinder plots and an optimal blend of essential oils determined. The optimum blend of essential oils was then formulated into a nanoemulsion using the self-emulsification technique. The antimicrobial potential of the nanoemulsed essential oil formulation was validated by means of MIC analysis against the same nine micro-organisms and the results of the formulation compared to those of the pure essential oils in optimal ratios.

This study confirmed the antimicrobial potential of essential oils, with noteworthy inhibition observed for 31.9% of essential oils against all the pathogens tested. *Amyris balsamifera* L. (amyris), *Coriandrum sativum* L. (coriander) and *Cinnamomum zeylanicum* Blume (ceylon cinnamon) showed the broadest spectrum of activity. Computational software was applied to define the chemistry responsible for individual antimicrobial effect. Eugenol was identified as the most frequently reoccurring biomarker that contributed to broad-spectrum noteworthy antimicrobial activity of essential oils; while linalyl acetate, α-pinene and β-pinene were frequently responsible for poor antimicrobial activity. The chemometric tool was assessed for prediction accuracy by means of antimicrobial validation. The prediction accuracy relating to the chemometric outcomes of this study was 92.3% for active biomarker predictions and 61.9% for inactive biomarker predictions. Following the analysis of individual essential oils, combinations were assessed for antimicrobial potential in 1:1 and varying ratios. The prediction tool, SynergyFinder was implemented and compared to isobolograms generated for essential oil blends. Prediction accuracy for synergy by SynergyFinder was on average 34% across all nine ratios studied. This low outcome is primarily due to differences in mathematical theory applied to the definition of synergy between the isobologram and SynergyFinder. An optimal blend of essential oil was identified. This blend included the *Hyssopus officinalis* var. *angustifolius* (M.Bieb.) Benth. (hyssop) in combination with *Rosmarinus officinalis* var. *angustifolius* (Mill.) DC. (rosemary) essential oil in blends of 49.57% of *H. officinalis* to 50.43% of *R. officinalis*. This blend of essential oil was developed into a pharmaceutical nanoemulsion formulation and assessed against the same panel of pathogens. The formulation provided a six-fold enhanced antimicrobial effect as compared to the neat essential oil blend.

Essential oils, as investigated here, display antimicrobial potential alone and in combination against pathogens of the respiratory tract. Chemometric analysis was proven to be an effective

tool for identifying active compounds that could potentially become novel lead compound for new antimicrobials. The blended essential oils further demonstrated potential for holistic treatment effects, proving anti-inflammatory and non-toxic activity when equally blended. It was further determined that the SynergyFinder prediction software, critical in preclinical formulation design, provides valuable supporting tools to isobolograms when optimizing essential oil combinations for antimicrobial effect. This study has also gone on to show the opportunity to combine essential oils into novel pharmaceutical formulations for enhanced antimicrobial effects. Therefore, this study has been majorly successful in identifying unique resources for antimicrobial optimization of essential oils and creates an exciting starting point for future research in this field.

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Deuteronomy 2:7, "For the blessing of the Lord your God has been on you in all the work of your hands"

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List of equations

Equation 1:

The ΣFIC values of the combinations were calculated using the following equations;

$$\text{FIC (i)} = \frac{\text{MIC of (a*) combined with (b*)}}{\text{MIC of (a) independently}}$$

$$\text{FIC (ii)} = \frac{\text{MIC of (b) combined with (a)}}{\text{MIC of (b) independently}}$$

*Where (a) is the MIC of the one oil in the combination and (b) is the MIC of the other.

From these values the FIC index will be calculated as: ΣFIC = FIC (i) + FIC (ii).

Equation 2:

The percentage mortality associated to the brine shrimp assay was calculated using the following equation;

$$\%\text{mortality} = \frac{\text{dead shrimp at 24 hours (or 48 hours) (before acetic acid)-dead shrimp (time 0)}}{\text{dead shrimp (after acetic acid)}}\ x\ 100$$

List of abbreviations

%	Percent
°C	Degree celsius
µg	Micrograms
µl	Microliters
AA	Arachidonic acid
AD	Agar diffusion
ADM	Agar diffusion method
ATCC	American type culture collection
CAM	Complementary and alternative medicine
CC	Cytotoxic concentration
CFU	Colony forming units
CLSI	Clinical and laboratory standards institute
CTA	Contact time assay
DD	Disc diffusion
DIY	Do it yourself
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
EO	Essential oil
EPA	Eicosapentaenoic acid
ESBL	Extended spectrum β-lactamase
ETA	Eicosatrienoic acids
FDA	Food and drug administration
G-	Gram
GC-MS	Chromatography and mass spectrometry
GC-MS/FID	Gas chromatography mass spectrometry/flame ionization detector
GRAS	Generally recognized as safe
IC	Inhibitory concentration
IL	Interleukin
INT	*p*-Iodonitrotetrazolium violet
LPS	Lipopolysaccharides
m/z	Mass-to-charge ratio

MAC	Microdilution method
MFBF	Collection of microorganisms of the department of microbiology
Mg	Milligram
MIC	Minimum inhibitory concentration
ml	Milliliters
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
n.m	Non-mentioned
NaOH	Sodium methoxide
NCCLS	National Committee for Clinical Laboratory Services
NCR	No composition results reported
ND	Not determined
NHLS	National Health Laboratory Service
NI	No inhibition
OM	Outer membrane
OPLS-DA	Orthogonal projection to latent structures discriminant analysis
PCA	Principal component analysis
PG	Propylene glycol
PGE	Prostaglandin E_2
RCT	Randomised controlled trials
RTM	Retention time mass
SAR	Structure activity relationship
Spp.	Species
TB	Tuberculosis
TGB	Thioglycolate broth
TSA	Tryptone soya agar
TSB	Tryptone soya broth
USA	United States of America
v/v	Volume per volume
w/v	Weight per volume
WHO	World Health Organisation
ΣFIC	Fractional inhibitory concentration index

Preview and description of thesis

This study is a thesis by publication that incorporates research published into chapters; with permissions acquired from the publishers for the re-use in this thesis available in **Appendix B**. **Figure 1** is a flow chart that demonstrates the sequence of events followed in undertaking this research. The design of this thesis has been divided into four parts. Firstly (shown in red), the essential oils recommended for various respiratory tract infections were identified and reviewed **(Chapter 1, Article 1)**. The essential oils were then investigated for antimicrobial effect against nine micro-organisms of the respiratory tract (*Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Mycobacterium smegmatis*, *Klebsiella pneumoniae*, *Moraxella catarrhalis*, *Haemophilus influenzae*, *Cryptococcus neoformans*). These essential oils were further analysed using gas chromatography coupled to mass spectrometry (GC-MS), the major compounds identified, and purity confirmed. Using multivariate tools such as chemometric analysis, the compounds responsible for the noteworthy antimicrobial activity against the respective pathogen were identified **(Chapter 2, Article 2)**. This was followed by the investigation of the selected 369 essential oil combinations for antimicrobial, anti-inflammatory and toxic effects **(Chapter 3, Article 3)**. This chapter could thus provide credence to the displayed antimicrobial activity of essential oils (alone and in combination) responsible for antimicrobial activity. Next (following the refinement of samples), five essential oil combinations were defined as the most promising for antimicrobial, anti-inflammatory and non-toxic effects and were assessed for antimicrobial activities when blended in varying ratios **(Chapter 3, Article 4)**. Isobolograms and synergy contour maps (Synergy Finder) were created to define optimized synergy in each blend **(Chapter 3, Article 4)**. Lastly (shown in blue), a best combination of essential oils was blended according to optimized synergy results and developed into a solution based pharmaceutical formulation for inhalation delivery in the management of respiratory tract infections (**Chapter 4, Article 5**). **Chapter 5** provides a final overview and conclusion.

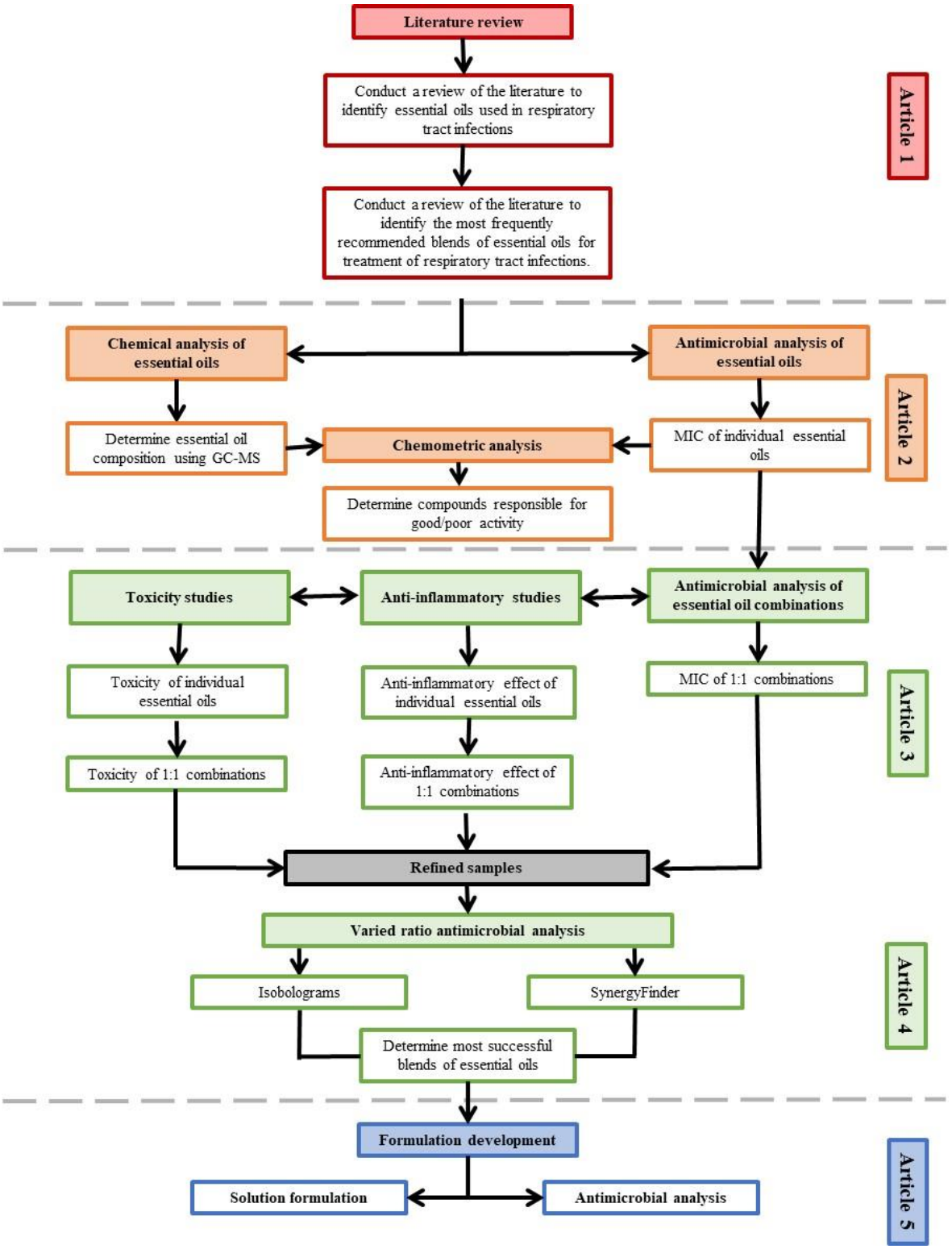


Figure 1: Schematic outlay of thesis

CHAPTER 1

GENERAL INTRODUCTION



1. Background

1.1. Essential oils

Essential oils are odorous and volatile liquids derived from plant material. Plants produce essential oils as secondary metabolites which are stored within the plant in secretory structures such as glands, secretory hairs, secretory ducts, secretory cavities or resin ducts (Djilani and Dicko, 2012). Essential oils play an important role in nature where they act to protect the plant by means of antibacterial, antiviral, antifungal and insecticidal action. In addition, essential oils protect the plant from herbivores while attracting some insects to scatter plant pollen and seeds for plant reproduction (Bakkali et al., 2008). Essential oils are commonly obtained by hydrodistillation, and sometimes by solvent extraction, or supercritical fluid extraction (Bakkali et al., 2008, Ni et al., 2021). Essential oils are composed predominantly of mono- and sesquiterpenes, but other types of compounds such as allyl and isoallyl phenols may also be present within an essential oil (Ríos, 2016). The applications of essential oils are equally as diverse with essential oils having been applied in cosmetics and perfumes, as well as medicinal applications due to their perceived therapeutic benefits (Djilani and Dicko, 2012).

1.2.Essential oils as antimicrobial agents

Essential oils have been used for their antimicrobial potential as early as 4500 BC, where the Ancient Egyptians made use of these oils in cosmetics and ointments for medicine (Başer and Buchbauer, 2009). As of 1937, Rene Gattefosse, developed the term "Aromatherapy" and began researching essential oils for their therapeutic properties (Bakkali et al., 2008). The clinical use of essential oils continued to develop in France where in 1969; Maurice Girault created the 'aromatogram' (Peace Rhind, 2012). Aromatograms, similar to clinical antibiograms, list the essential oils identified as having antimicrobial potential against an individual microbial pathogen, as determined in laboratory settings. As a result of this work, and others by Daniel Penoel, Jean Valnet and Pierre Franchomme, the use of essential oils for treating infections has developed into a clinical practice of aromatherapy which is known today as 'aromatic medicine' (Peace Rhind, 2012). The practice of aromatherapy and aromatic medicine remains one of the most popular forms of complementary medicine to date (El-Anssary, 2020). Despite the empirical knowledge held for thousands of years concerning the antimicrobial potential of essential oils, clinical research performed in laboratories around the world have further proven their potential, advancing the interest in essential oil use within

clinical settings. Numerous scientific studies and reviews performed over the last four decades have highlighted the potential of essential oils for antimicrobial effect (Deans and Ritchie, 1987, Burt, 2004, van Vuuren and Viljoen, 2011, de Rapper et al., 2012, Lang and Buchbauer, 2012, Raut and Karuppayil, 2014, Orchard et al., 2018a, Ni et al., 2021).

For human application, essential oils are applied through topical, oral and inhalation in aromatic medicine practice. Topical application includes the massage or application of diluted essential oils to the surface of the skin. According to Tisserand and Young (2014), essential oils applied through this route of administration have poor absorption with only 5% of the applied essential oil transported into the bloodstream. Essential oils can also be administered via ingestion along the oral route. These essential oils are encapsulated within gelatin capsules or diluted in a glass of water for administration along this route. Bioavailability of essential oil is indicated at 95% via oral ingestion, however, the consumption of these products is leveled with a degree of uncertainty with regards to safety and as such not strongly advocated (Tisserand and Young, 2014). Lastly, essential oils can be administered through the method of inhalation. Inhalation of essential oils is the action of direct breathing in of these agents for therapeutic effect. Essential oils, once inhaled, have direct distribution to the lungs, with an effective bioavailability of 50% (Tisserand and Young, 2014). Essential oils, once inhaled, are able to reach the tiniest spaces within the lungs and due to their effects on mucocilliary clearance, are able to remain in these spaces for prolonged periods (Hasani et al., 2003). Inhalation of essential oils can also result in changes to behavior and mood when treating patients, as 5% of essential oils administered via inhalation are transported to the brain by means of olfactory receptors. Commercially available aromatherapeutic texts note extensively the use of essential oils by means of inhalation for the management of respiratory related infections, with numerous texts and internet sources available for quick reference of treatment regimens.

1.3. The use of essential oils in the respiratory tract

According to the European Pharmacopoeia, more than 25 essential oils are listed as ingredients for use in the respiratory tract in the management of respiratory related infections. These essential oils include *Pimpinella anisum* L., *fructus* (anise), *Foeniculi amari fructus* (bitter fennel), *Eucalyptus globulus* Labill. (eucalyptus), *Menthae piperitae aetheroleum* (peppermint), *Melaleuca alternifolia* Cheel. (tea tree) and *Thymus vulgaris* L. (thyme). Commercially available aromatherapeutic texts, however, note a greater number of essential

oils for use in the respiratory tract (Lawless, 1995, Shealy, 1998, Hili, 2001, Sellar, 2005, Clarke, 2009, Buckle, 2014, Curtis et al., 2016). The physiological effects of individual essential oils and their components on the respiratory system has been studied *in vitro* and in animal models. Studies have demonstrated that some essential oils cause a reduction in respiratory tract infection symptoms (Kamin and Kieser, 2007, Shields, 2011). Despite the potential of these essential oils to manage symptoms associated with respiratory infection, risk exists (respiratory depression, increased mucous production and decreased mucocillary beat) when applied in the respiratory tract (Plotkowski et al., 1993, Su et al., 1993, Sekizawa et al., 1996). In addition, little is known of the effect of essential oils on pathogens clinically relevant to respiratory tract infections. Despite the lack of scientific support for the use of essential oils for respiratory infections, a large array of resources exist regarding the use of essential oils for these purposes. The recommendation and claims of use for many of the essential oils may have been from knowledge passed down over the generations, or may be due to the information taken from the known uses of the herbs or extracts (Orchard et al., 2018b). This further indicates why scientific validation for the use of essential oils against respiratory tract associated pathogens is necessary.

1.4. The identification of commercial essential oils used in the respiratory tract

Essential oils recommended for use in the respiratory tract were identified using the aroma-therapeutic literature available to the layman (Lawless, 1995, Shealy, 1998, Hili, 2001, Sellar, 2005, Clarke, 2009, Buckle, 2014, Curtis et al., 2016). Review of the literature allowed for the identification of 55 commercial essential oils cited for use in the respiratory tract for the management of respiratory infections. An overview of the essential oils identified is provided in Table 1.1 with a summary of the respiratory uses of these essential oils provided in Figure 1.1 (also present in the literature review Leigh-de Rapper and van Vuuren (2020)).

Table 1.1: Summary of individual essential oils indicated in the management of respiratory tract conditions

Scientific name	Common name	Indication for use*
Abies balsamea	Fir	Cold, bronchitis, congestion, mucous reduction, flu
Amyris balsamifera	Amyris	Asthma, cold, cough, bronchitis, flu
Angelica archangelica	Angelica	Cold, bronchitis, pleurisy

Scientific name	Common name	Indication for use*
Boswellia carterii	Frankincense	Asthma, catarrh, cough, laryngitis, bronchitis, congestion, mucous reduction
Canarium luzonicum	Elemi	Catarrh, cough, bronchitis, congestion, mucous reduction
Carum carvi	Caraway	Laryngitis, bronchitis, congestion, mucous reduction
Cinnamomum camphora	Camphor	Cold, cough. pneumonia
Cinnamomum zeylanicum	Ceylon cinnamon	Cold, flu
Citrus aurantifolia	Lime	Catarrh, cough, sinusitis, bronchitis, congestion, mucous reduction
Citrus sinensis	Orange	Cold, bronchitis
Citrus bergamia	Bergamot	Bronchitis, TB, tonsillitis
Citrus limonum	Lemon	Cold, cough, laryngitis, pharyngitis, bronchitis, flu
Commiphora molmol	Myrrh	Catarrh, cold, cough, pharyngitis, bronchitis, congestion, mucous reduction
Commiphora myrrha		
Coriandrum sativum	Coriander	Flu
Cupressus sempervirens	Cypress	Cold, diphtheria, bronchitis, flu, hay fever
Cymbopogon citratus	Lemon Grass	Laryngitis
Cymbopogon martinii		
Daucus carota	Carrot Seed	Cough, bronchitis, congestion, mucous reduction
Elettaria cardamomum	Cardamom	Flu
Eucalyptus globulus	Eucalyptus	Catarrh, cold, sinusitis, laryngitis, pharyngitis, bronchitis, pneumonia, flu
Ferula galbaniflua	Galbanum	Cough, congestion, mucous reduction
Foeniculum vulgare	Fennel	Cough
Helichrysum officinalis	Immortelle	Asthma, cold, cough, bronchitis, congestion, mucous reduction, flu
Helichrysum racemosa		
Hyssopus officinalis	Hyssop	Asthma, cold, bronchitis, congestion, mucous reduction, flu, sore throat
Illicium verum	Star anise	Cough
Jasminum officinalis	Jasmine	Cold, cough, laryngitis, pharyngitis, bronchitis, pneumonia
Juniperus virginiana	Cedarwood	Catarrh, cough, bronchitis, congestion, mucous reduction
Laurus nobilis	Bay	Bronchitis

Scientific name	Common name	Indication for use*
Lavandula angustifolia	Lavender	Catarrh, cough, laryngitis, bronchitis, pneumonia, TB
Lavandula burnati	Lavandin	Laryngitis, bronchitis, flu
Lavandula latifolia		
Lavandula spicia	Lavender Spike	Laryngitis, bronchitis
Litsea cubeba	May Chang	Oral thrush
Melaleuca alternifolia	Tea-Tree	Catarrh, cold, flu
Melaleuca cajeput	Cajeput	Asthma, cold, sinusitis, laryngitis, pharyngitis, bronchitis, flu
Melaleuca viridiflora	Niaouli	Asthma, catarrh, cold, cough, sinusitis, laryngitis, pharyngitis, bronchitis, pneumonia, TB, congestion, mucous reduction
Mentha piperita	Peppermint	Asthma, cough, sinusitis, bronchitis, flu
Myrtus communis	Myrtle	Congestion, mucous reduction
Ocimum basilicum	Basil	Catarrh, cough, flu
Origanum majorana	Marjoram	Asthma, cold, sinusitis, bronchitis
Origanum vulgare	Origanum	Cold, bronchitis, flu
Pimenta officinalis	Pimento	Cold, bronchitis, flu
Pimenta racemosa		
Pimpinella anisum	Aniseed	Cold, congestion, mucous reduction
Pinus sylvestris	Pine	Asthma, cold, cough, laryngitis, bronchitis
Piper nigrum	Black Pepper	Catarrh, cough, flu
Rosa damascena	Rose	Cough, laryngitis
Rosmarinus officinalis	Rosemary	Asthma, cold, cough, bronchitis, flu
Salvia officinalis	Sage	Catarrh, cold, bronchitis, flu
Salvia sclarea	Clary sage	Asthma, cough
Santalum album	Sandalwood	Cough, laryngitis, bronchitis
Santalum austrocaledonicum		
Styrax benzoin	Benzoin	Cold, cough, sinusitis, laryngitis, pharyngitis, bronchitis, pneumonia, congestion, mucous reduction, flu
Syzygium aromaticum	Clove	Asthma, cold, bronchitis, TB, flu
Syzygium caryophyllata		
Tagetes minuta	Tagetes	Oral thrush
Tagetes patula		
Thymus vulgaris	Thyme	Asthma, cold, cough, laryngitis, pharyngitis, bronchitis, flu
Tilia europaea	Linden Blossom	Bronchitis, flu, pleurisy
Viola odorata	Violet	Diphtheria, laryngitis, congestion, mucous reduction
Zingiber officinale	Ginger	Cough, flu

*References include Lawless, 1995, Shealy, 1998, Hili, 2001, Sellar, 2005, Clarke, 2009, Buckle, 2014, Curtis et al., 2016

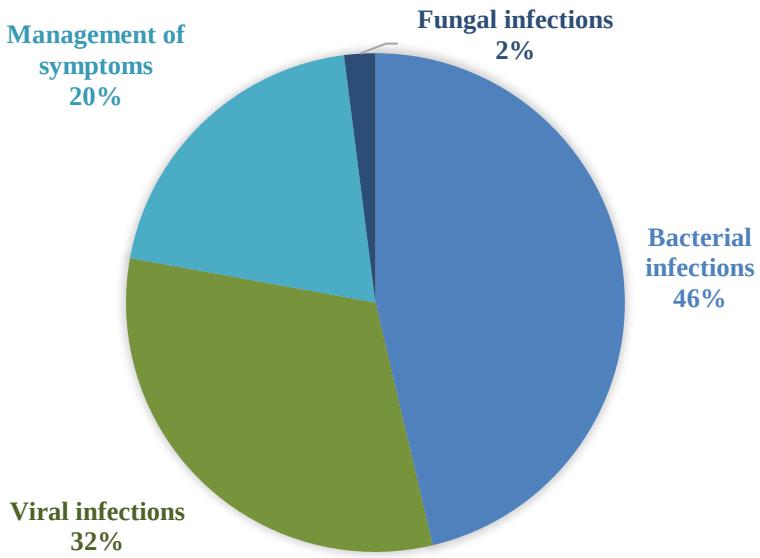


Figure 1.1: Summary of categorized respiratory conditions in which essential oils are used.

Upon identification of the individual essential oils indicated for use in the respiratory tract, it was noted that blends comprising more than one essential oil was commonly indicated. Essential oil combinations are the preferred means of essential oil application in aromatic medicine due to the perceived synergistic and holistic benefits offered by this approach (Lawless, 1995). Therefore, following the identification of the individual essential oils indicated in the management of respiratory infections, further investigation was required to determine the essential oils indicated for use in combination for an enhanced therapeutic effect. Using the same resources, Table 1.2 summarises the possible combinations that could be identified for each of the 55 essential oils. Further detail into the combinations is discussed in Chapter 3.

Table 1.2: Summary of recommended essential oil combinations

Scientific name	Common name	Use in combination*
Abies balsamea	Fir	Basil, caraway, cedarwood, frankincense, lavender, myrtle, niaouli
Amyris balsamifera	Amyris	Benzoin, elemi, frankincense, galbanum, jasmine, lavender spike, sage
Angelica archangelica	Angelica	Basil, benzoin, hyssop, lavender, lemon, sage, sandalwood
Boswellia carterii	Frankincense	Basil, bergamot, black pepper, cardamom, cedarwood, cinnamon, galbanum, lavender, myrrh, orange, pine, sandalwood

Scientific name	Common name	Use in combination*
Canarium luzonicum	Elemi	Cardamom, frankincense, galbanum, ginger, lavender, orange, rosemary
Carum carvi	Caraway	Basil, bay, benzoin, cardamom, coriander, elemi, frankincense, galbanum, ginger, lavender, orange
Cinnamomum camphora	Camphor	Basil, cajeput, lavender
Cinnamomum zeylanicum	Ceylon cinnamon	Basil, benzoin, cardamom, clove, coriander, eucalyptus, frankincense, galbanum, ginger, lavender, lemon, myrrh, orange, pine, rosemary, thyme
Citrus aurantifolia	Lime	Angelica, bergamot, black pepper, carrot seed, ginger, lavender, linden, rose, rosemary, sage, tea-tree
Citrus sinensis	Orange	Angelica, black pepper, caraway, carrot seed, cinnamon, clove, coriander, cypress, frankincense, ginger, hyssop, jasmine, lavender, lemon, marjoram, niaouli, rose, rosemary, sage, tagetes, thyme
Citrus bergamia	Bergamot	Coriander, cypress, eucalyptus, jasmine, lavender, lemon, marjoram, myrrh, pine, rosemary, sandalwood, tea-tree
Citrus limonum	Lemon	Benzoin, cardamom, carrot seed, cedarwood, cinnamon, eucalyptus, fennel, frankincense, ginger, hyssop, lavender, linden, myrtle, niaouli, orange, peppermint, rose, sandalwood, tagetes, thyme
Commiphora molmol	Myrrh	Benzoin, cedarwood, cinnamon, clove, cypress, eucalyptus, frankincense, galbanum, lavender, lemon, peppermint, pine, sandalwood, tagetes
Commiphora myrrha		
Coriandrum sativum	Coriander	Bergamot, black pepper, cardamom, cinnamon, cypress, frankincense, galbanum, ginger, jasmine, lemon, orange, pine, sage, sandalwood
Cupressus sempervirens	Cypress	Bergamot, cedarwood, clary sage, frankincense, lavender, lemon, marjoram, orange, pine, rosemary, sage, sandalwood
Cymbopogon citratus	Lemon Grass	Basil, cedarwood, clove, coriander, eucalyptus, jasmine, lavender, niaouli, orange, peppermint, rosemary, sandalwood, tagetes, thyme
Cymbopogon martinii		
Daucus carota	Carrot Seed	Bergamot, lavender, lemon, lime, orange, rosemary
Elettaria cardamomum	Cardamom	Coriander, frankincense, galbanum, lemon, myrtle, pine

Scientific name	Common name	Use in combination*
Eucalyptus globulus	Eucalyptus	Benzoin, cedarwood, coriander, cypress, lavender, lemon, lemongrass, marjoram, peppermint, pine, rosemary, thyme, tea-tree
Ferula galbaniflua	Galbanum	Cardamom, elemi, frankincense, ginger, jasmine, pine, rose, tagetes
Foeniculum vulgare	Fennel	Basil, lavender, lemon, rose, rosemary, sandalwood
Helichrysum officinalis *Helichrysum racemosa*	Immortelle	Bergamot, frankincense, lavender, orange, rose
Hyssopus officinalis	Hyssop	Angelica, cypress, fennel, lavender, lemon, orange, rosemary, sage, sandalwood
Illicium verum	Star anise	Cardamon, caraway, coriander, cypress, fennel, ginger
Jasminum officinalis	Jasmine	Bergamot, frankincense, immortelle, lavender, orange, rose, sage, sandalwood
Juniperus virginiana	Cedarwood	Benzoin, bergamot, cinnamon, cypress, frankincense, jasmine, lavender, lemon, linden, myrrh, rose, rosemary, sandalwood
Laurus nobilis	Bay	Cedarwood, coriander, eucalyptus, ginger, lavender, lemon, marjoram, orange, rosemary, thyme
Lavandula angustifolia	Lavender	Bay, bergamot, caraway, carrot seed, cedarwood, cinnamon, clary sage, clove, hyssop, jasmine, lemon, marjoram, myrtle, niaouli, orange, pine, rosemary, tagetes, thyme
Lavandula burnati *Lavandula latifolia*	Lavandin	Bergamot, clary sage, immortelle, jasmine, lemon, orange
Lavandula spicia	Lavender Spike	Bergamot, immortelle, jasmine, lemon, orange
Litsea cubeba	May Chang	Basil, jasmine, lavender, orange, rose, rosemary
Melaleuca alternifolia	Tea-Tree	Cinnamon, clary sage, clove, coriander, cypress, elemi, eucalyptus, fennel, fir, frankincense, galbanum, garlic, ginger, hyssop, immortelle, jasmine, lavandin, lavender, lavender spike, lemon, lemongrass, lime, linden, marjoram, myrrh, myrtle, niaouli, orange, origanum, peppermint, pine, rose, rosemary, sage, sandalwood, tagetes
Melaleuca cajeput	Cajeput	Angelica, bergamot, cardamom, clove, eucalyptus, hyssop, immortelle, lavender, lemon, myrtle, niaouli, pine, rose, rosemary, sandalwood, thyme

Scientific name	Common name	Use in combination*
Melaleuca viridiflora	Niaouli	Basil, coriander, fennel, frankincense, galbanum, lavender, lemon, myrrh, orange, peppermint, pine, rosemary, sage
Mentha piperita	Peppermint	Benzoin, cedarwood, cypress, eucalyptus, ginger, lavender, lemon, marjoram, myrtle, niaouli, pine, rosemary, sandalwood, tagetes
Myrtus communis	Myrtle	Bergamot, cardamom, coriander, lavender, lemon, lemongrass, peppermint, rosemary, tagetes, thyme
Ocimum basilicum	Basil	Bergamot, black pepper, cedarwood, eucalyptus, hyssop, lavender, lime, marjoram, rosemary, sage, sandalwood, tea-tree
Origanum majorana	Marjoram	Bergamot, cedarwood, cypress, eucalyptus, frankincense, lavender, orange, rosemary, sandalwood, thyme
Origanum vulgare	Origanum	Angelica, basil, fennel, lemongrass, myrrh, pine, rosemary, tagetes
Pimenta officinalis	Pimento	Frankincense, galbanum, ginger, lavender, lemon, lemongrass, orange, pine
Pimenta racemosa		
Pimpinella anisum	Aniseed	Amyris, cardamon, caraway, cedarwood, coriander, fennel, galbanum
Pinus sylvestris	Pine	Cardamom, cedarwood, cinnamon, clove, cypress, eucalyptus, galbanum, lavender, lemon, myrrh, myrtle, niaouli, rosemary, sandalwood, tagetes, thyme
Piper nigrum	Black Pepper	Basil, benzoin, bergamot, cedarwood, cypress, frankincense, lavender, lemon, marjoram, rosemary, sandalwood
Rosa damascena	Rose	Bergamot, clary sage, galbanum, jasmine, lavender, orange, sandalwood
Rosmarinus officinalis	Rosemary	Basil, bergamot, black pepper, carrot seed, cedarwood, cinnamon, clove, cypress, frankincense, ginger, hyssop, lavender, lemongrass, marjoram, myrrh, niaouli, orange, peppermint, pine, tagetes
Salvia officinalis	Sage	Bay, bergamot, coriander, frankincense, ginger, hyssop, jasmine, lavender, myrrh, niaouli, orange, rosemary, sandalwood, tagetes, thyme
Salvia sclarea	Clary sage	Bergamot, cedarwood, cypress, frankincense, jasmine, lavender, lime, sandalwood
Santalum album	Sandalwood	Basil, benzoin, bergamot, black pepper, cedarwood, cypress, frankincense, ginger, hyssop, jasmine, lavender, lemon, marjoram, myrtle, rose, thyme
Santalum austrocaledonicum		

Scientific name	Common name	Use in combination*
Styrax benzoin	Benzoin	Bergamot, cardamom, coriander, cypress, frankincense, jasmine, lavender, lemon, myrrh, orange, rose, sandalwood
Syzygium aromaticum	Clove	Basil, benzoin, bergamot, black pepper, cedarwood, cinnamon, eucalyptus, lavender, lemon, orange, peppermint, rosemary, thyme, tea-tree
Syzygium caryophyllata		
Tagetes minuta	Tagetes	Galbanum
Tagetes patula		
Thymus vulgaris	Thyme	Bergamot, black pepper, cedarwood, cinnamon, clove, eucalyptus, ginger, lavender, lemon, lemongrass, myrtle, niaouli, orange, pine, rosemary, sage, thyme
Tilia europaea	Linden Blossom	Benzoin, cedarwood, ginger, jasmine, lavender, rose, tea-tree
Viola odorata	Violet	Benzoin, frankincense, jasmine, lavender, lemon, orange, rose, sandalwood
Zingiber officinale	Ginger	Bay, cajeput, cardamom, caraway, cedarwood, cinnamon, clove, coriander, elemi, eucalyptus, frankincense, lemon, lime, myrtle, orange, peppermint, rosemary, sandalwood, thyme

*References include Lawless, 1995, Shealy, 1998, Hili, 2001, Sellar, 2005, Clarke, 2009, Buckle, 2014, Curtis et al., 2016; Common name only cited

The use of essential oils in combination requires consideration that there is potential that the blend may result in synergistic or even antagonistic interactions. The aim of combining essential oils is to provide an enhanced effect and thus the resultant combination should act synergistically when blended. Synergy can be defined as an effect seen by a combination of substances being greater than would have been expected from individual contributions (Williamson, 2001). Synergy is commonly sought in the pharmaceutical profession when aiming to produce new and more effective drug combination formulations (e.g. clavulanic acid and amoxicillin). However, essential oils are not only blended for their antimicrobial potential when combined but also for possible synergy that results in enhanced anti-inflammatory and non-toxic effects. These interactions are explored and further elaborated on in Chapters 2 and 3. Current antimicrobial research is very effective in being able to identify the potential of single oils or 1:1 combinations for antimicrobial purposes; however, finite research exists for the combined effect of essential oils in varied ratios. Just as essential oils are seldom applied individually in aromatic medicine, so too are they rarely applied in equal ratios when used in combination for a therapeutic effect. More commonly, in aromatic practice essential oils are

combined in varying concentrations to illicit an effect that is dependent on the individual qualities of each oil in the combination (New Directions Aromatics, 2020). Although research considering the effect of essential oils in varied ratios is sparse, it has been consistently noted within the published data that when essential oils are mixed in various concentrations, that a varied antimicrobial interaction may occur depending on the concentration of the two components used (Foucquier and Guedj, 2015). Therefore, essential oil research for antimicrobial effectiveness should consider the use of these products in varied ratios to confirm and optimize the antimicrobial effects of an essential oil blend at a specific ratio. Optimization in the process of pharmaceutical development is an expectation for formulation design, as product optimization provides for improved patient safety and compliance, reduced unit cost, and reduced probability of a failed product (Taylor, 2015). However, despite the ability of current methods available for antimicrobial research to provide a more precise effective dose ratio when reviewing essential oil blends in various ratios as compared to equal blends, infinite ratios of essential oils in combination exist when only two essential oils are combined. Therefore large-scale systematic antimicrobial screenings would be required in order to identify the exact ratio within an essential oil combination for optimum antimicrobial synergy, which is not practical with the current methods available (Fatsis-Kavalopoulos et al., 2020). The use of computer-aided software to quantify synergy for drug design and development has been in use within the pharmaceutical industry for a number of years, with great success (Yu and MacKerell, 2017). The use of this technology in essential oil antimicrobial research is novel, with limited data available to validate its potential for use in this field of study. However, the application of this technology for antimicrobial determination amongst essential oils provides the possibility to test a vast array of essential oil combinations using a limited number of fixed ratio experiments to optimize these combinations for synergy. The use of this technology and the determination of essential oils interactions is explored and further elaborated on in Chapter 4.

In summary, both essential oils alone and in combination need to be validated in terms of efficacy and safety. This study predominantly focuses on addressing antimicrobial activity, anti-inflammatory activity and toxicity specifically related to essential oils combinations indicated for use in the respiratory tract. Knowledge regarding the optimal essential oil blends are then applied to the novel approach of synergy prediction using computer-aided technology as a means to optimize essential oil blends for formulation design for antimicrobial effect against pathogens relevant to the respiratory tract.

1.5. Aim and objectives of the thesis

The aim of this study is to investigate the accuracy of synergy prediction made by computer-aided software for the use of essential oils in the treatment of respiratory infections.

The objectives of this study are as follows:

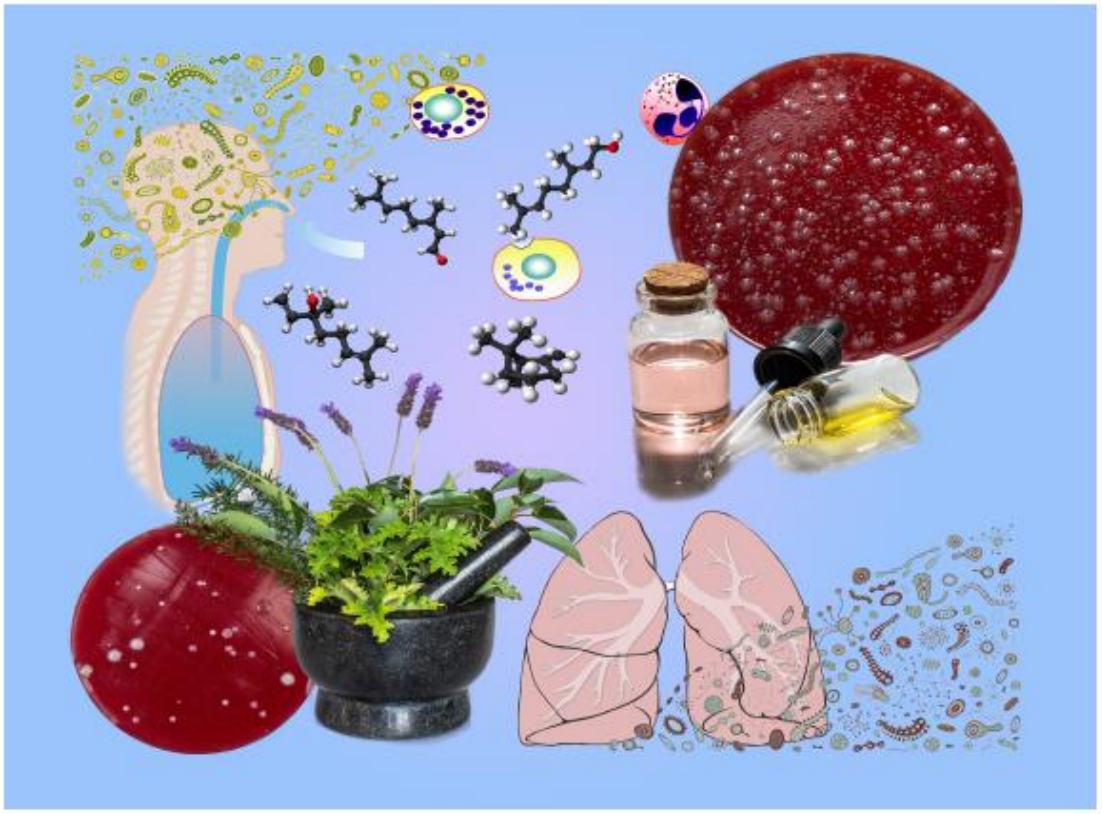
- To identify the essential oils most commonly used and recommended for the treatment of respiratory tract infections.
- To perform GC-MS analysis on the essential oils in order to identify the chemotype.
- To perform antimicrobial testing of each essential oil against the micro-organisms associated with respiratory infections using the minimum inhibitory concentration (MIC) assay.
- To perform structure activity relationship (SARS) of the components of the essential oils by means of chemometric analysis (SIMCA).
- To identify the most frequently documented blends that are used in treating respiratory infections.
- To determine the interactive profile of 1:1 combinations by calculating of the fractional inhibitory concentration (ΣFIC).
- To determine the interactive profile of 1:1 combinations for anti-inflammatory effects.
- To determine the interactive profile of 1:1 combinations for non-toxic effects.
- To determine optimized antimicrobial synergy from essential oil blends where synergy is observed in the ΣFIC assessment across antimicrobial, anti-inflammatory and toxicity studies, by means of various ratio studies (isobologram analysis).
- To perform SynergyFinder analysis to determine optimized antimicrobial synergy from essential oil blends where synergy is observed in the ΣFIC assessment across antimicrobial, anti-inflammatory and toxicity studies, by means of various ratio studies.
- To formulate an essential oil product based on an ideal and optimized essential oil mixture using the results of the various ratio studies.

Article 1 - Literature review on essential oils used in the respiratory tract

At the time of this thesis, this article had been published. This article is shown as it appears in publication. The article featured as the cover page for the volume.

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REVIEW

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Odoriferous Therapy: A Review Identifying Essential Oils against Pathogens of the Respiratory Tract

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This review explores the body of scientific information available on the antimicrobial properties of essential oils against pathogens responsible for respiratory infections and critically compares this to what is recommended in the Layman's aroma-therapeutic literature. Essential oils are predominantly indicated for the treatment of respiratory infections caused by bacteria or viruses (total 79.0%), the efficacy of which has not been confirmed through clinical trials. When used in combination, they are often blended for presumed holistic synergistic effects. Of the essential oils recommended, all show some degree of antioxidant activity, 50.0% demonstrate anti-inflammatory effects and 83.3% of the essential oils showed antihistaminic activity. Of the essential oils reviewed, 43.8% are considered non-toxic while the remaining essential oils are considered slightly to moderately toxic (43.7%) or the toxicity is unknown (12.5%). Recommendations are made for further research into essential oil combinations.

Keywords: respiratory tract, essential oils, antimicrobial properties, aromatherapy, combination, drug discovery, natural products.

Introduction

Respiratory tract infections have been the cause of up to 10.8 million child deaths annually,[1] with approximately 47.0% of all new hospital admissions in pediatric patients being reported.[2] The bacterial micro-organisms most commonly associated with respiratory related infections include *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus*.[3] *Streptococcus pneumoniae* is currently the leading cause of community-acquired pneumonia, bacterial meningitis, bacterial sinusitis and otitis media with resistance to antibiotics such as penicillins having developed over the last 20 years. Greater resistance of *S. pneumoniae* has spread to macrolides, doxycycline, trimethoprim-sulfamethoxazole and second and third generation cephalosporins.[4,5] Viruses are the leading cause of infections of the respiratory tract with pathogens such as the influenza virus and adenoviruses most commonly isolated. The socio-economic impact of these infections is substantial with excessive costs linked to treatment, hospitalization and absenteeism.

Essential oil blends are believed to work synergistically to heal the mind, body and senses.[6] These blends were frequently used to soothe aches and pains in ancient Egyptian medicine. More recently, blends such as *Pinus sylvestris* (pine), *Citrus limon* (lemon) and *Mentha piperita* (peppermint) are used to treat throat infections and congestion.[7] In a number of studies, it has been identified that practicing physicians have recommended Complementary and Alternative Medicines (CAM) such as essential oils to patients suffering from respiratory related infections.[8] These suggestions include the inhalation of essential oils to provide relief to congestion and aid in treatment. These suggestions are based on the assumption of safety associated with natural product therapies, believed to be beneficial for health with little or no adverse side effects.[9] This premise is unfounded, as many natural products have been identified as having potentially toxic effects.[10,11] The activity between blended molecules has the potential to increase side effects and promote or reduce toxicity. There is limited data on the effect of multiple essential oil combinations for toxicity. The aim of the review was to identify

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essential oils used for respiratory tract infections (according to the commercially available aroma-therapeutic texts) and then critically evaluate the available published literature to determine the potential of these essential oils as antimicrobial agents against respiratory tract infections.

Commercially available aroma-therapeutic texts were selected for review to determine the essential oils most commonly prescribed for use in respiratory tract infections. These texts included commonly cited and recommended, peer-reviewed publications. Websites were excluded from this analysis due to the lack of peer-review. Scientific literature for analysis was selected from three different electronic databases, namely PubMed, ScienceDirect, and Scopus. Literature was selected for works published during the period 2008–2019, with only studies relevant to respiratory related infections included for analysis. The filters used included either 'essential oils', 'volatile oils', 'aroma-therapy' or the scientific or common name for each individual essential oil listed in the aroma-therapeutic texts related to respiratory therapeutic potential. Additional filters included 'antimicrobial', 'antibacterial', 'antifungal', 'antiviral', 'respiratory', 'infection', 'combinations', 'fungal infections', 'bacterial infections', or 'toxicity'. To effectively identify the literature related to the use of essential oils for respiratory purposes, the inclusion criteria selected was broad with all published resources on the *in vitro* and *in vivo* bacterial and fungal effects of essential oils determined by means of the microdilution assay, macrodilution assay, or the agar dilution method considered. Literature identified



Professor Sandy van Vuuren I am currently employed in the Department of Pharmacy and Pharmacology and teach Pharmaceutical Microbiology at the University of the Witwatersrand. I hold an MSc in Medical Microbiology and a PhD undertaken on the antimicrobial properties of South African medicinal plants. My research interests include microbiological viability studies (medicinal plants and synthesized compounds), structure–activity relationships, pharmacological interactions, essential oil composition and antimicrobial efficacy as well as formulation studies involving antimicrobial principles. My role in postgraduate supervision not only extends to my own students (45 Postgraduate students graduated and currently supervising or co-supervising 16 students), but I am also involved in training students from universities such as University of Johannesburg, Tshwane University of Technology, University of Zululand, University of KwaZulu-Natal and as far afield as Cameroon. I have co-edited two special editions for the international journal 'Natural Product Communications' and presently serve on the editorial boards for 'The South African Journal of Botany' and 'Evidence-Based Complementary and Alternative Medicine'. Of the 144 ISI accredited publications authored, three have been awarded the best publication award in the years 2005, 2006 and 2019 at the International Conference on Pharmaceutical and Pharmacological Sciences. I have authored or co-authored over 225 conference contributions, many of which have been awarded prizes. I have been invited to give several keynote lectures at both local and international conferences. I currently hold a national benchmark evaluated rating (C1 NRF).



Ms. Stephanie Leigh-de Rapper I am a young Pharmacist and an emerging academic with expertise in Pharmaceutical Microbiology. I am currently employed in the Department of Pharmacy and Pharmacology as a lecturer in Pharmacy Practice at the University of the Witwatersrand. I hold a Master in Pharmacy in Pharmaceutical Microbiology and am currently undertaking a PhD on the use of essential oils in combination for the management and treatment of respiratory tract infections.

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in the review was excluded where lack of accessibility to the full publication was noted or techniques for analysis employed were other than those stated within inclusion criteria (that is, diffusion assays excluded due to the known variability obtained when examining results). Further exclusion criteria comprised of pathogens not relevant to the respiratory tract and its infections as well as studies investigating the potential of indigenous, non-commercial essential oils.

Infections of the Respiratory Tract

The respiratory system is a collection of structures that is required for breathing and consists of the upper and lower respiratory tracts. Structures that form the upper respiratory tract include the nose, nasal cavity, mouth, pharynx and larynx; while the lower respiratory tract includes the trachea, bronchi, bronchioles and alveoli.[12] The main function of the respiratory tract is to take in oxygen and release carbon dioxide, through a process known as respiration. Respiration does not occur in the entire respiratory tract but rather occurs at the level of the alveoli where gaseous exchange of oxygen and carbon dioxide occur. Oxygen is exchanged for carbon dioxide in the bloodstream, and then transported through the body to organs and structures to maintain functionality. The respiratory tract has natural barriers to prevent against infection such as nasal hair, mucociliary cells, phagocytic inflammatory cells and residual microbiota.[13] Micro-organisms that are inhaled must pass through these barriers to be pathogenic to the host. This breach in protection causes damage to related tissue, releasing the hormone histamine and initiating the inflammatory response.[12] Histamine is released by master immunity regulators known as mast cells in the area of tissue damage, facilitating the attraction of leukocytes and proteins to the site of injury. The attracted leukocytes proliferate at the site of tissue damage, destroying the pathogen.[12] The proliferation of leukocytes also results in the accumulation of fluid associated with vasodilation. This accumulated fluid is slowly drained by the lymphatic system as the infectious micro-organism is eradicated. The main functions of the response are to prevent spread of, as well as remove the micro-organisms responsible for the infection while promoting the repair of tissue. Respiratory complications such as sinusitis, bronchitis, bronchiolitis, laryngitis and pharyngitis are conditions where the inflammatory responses have been triggered in the sinuses, bronchi, bronchioles, larynx and pharynx respectively. The characteristic symptoms of these conditions, such as mucus production, pain and coughing are the result of the inflammatory response being activated.

Respiratory tract infections are among the leading causes of mortality,[1] with respiratory infections identified within 92.0% of all hospital admitted patients.[14] The micro-organisms most commonly associated with respiratory related infections are summarized in *Tables 1* and *2*.

Infections involving the respiratory tract are most often because of bacterial and viral pathogens and can be divided into two categories – upper and lower respiratory tract infections. Upper respiratory tract infections (URTI), are defined as acute infections of the nose, throat, larynx and trachea[18] and include conditions such as the common cold, acute sinusitis, pharyngitis, epiglottitis, acute rhinosinusitis and otitis media.[19] Pathogens most commonly associated with URTI include *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* (*Table 1*). Streptococci are estimated to cause 700 million human infections each year worldwide and are responsible for up to 500'000 deaths.[20] *Streptococcus pyogenes* has generally been associated with 'strep throat' and is commonly linked with infections such as pharyngitis, laryngitis and epiglottitis. *Streptococcus pneumoniae* is currently the leading cause of community-acquired pneumonia, bacterial meningitis, bacterial sinusitis and otitis media.[4,21] *Streptococcus pyogenes* and *S. pneumoniae* are Gram-positive bacteria that form part of the natural flora of the upper respiratory tract in healthy individuals. The structure of the bacteria allows for this class of micro-organism to evade detection by the host immunity at first stage of infection and therefore often results in severe life-threatening complications.[22] *Haemophilus influenzae* is a Gram-negative coccobacillus commonly detected within the respiratory tract of 75.0% of healthy adults. The incidence of upper respiratory tract infections associated to non-type *H. influenzae* has steadily increased since 1993 with infections such as sinusitis, otitis media and bronchitis associated to this pathogen.[22,23] Infections due to *Moraxella catarrhalis* invasion have emerged over the last 30 to 40 years, and are considered a serious cause of infection among healthy individuals.[24] *Moraxella catarrhalis* is a Gram-negative, diplococcus identified as natural flora within the respiratory tract of healthy individuals. Infections associated to *M. catarrhalis* include otitis media and bronchitis with pneumonia identified in immunocompromised hosts.[25,26]

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Table 1. Pathogens responsible for upper respiratory tract infections.

Infection of the upper respiratory tract	Clinical manifestations[a]	Most common pathogens associated with infection[b][c]		
		Bacterial	Viral	Fungal
Acute mastoiditis	Skin covering the mastoid process is red, swollen, and tender; external ear is pushed sideways and down, fever, pain around and within the ear, purulent, profuse discharge from the ear, hearing loss	*Streptococcus pyogenes*	NA[d]	NA
Acute sinusitis	Edematous and hyperemic nasal mucosa, malaise, low grade fever, as well as pain, sensation of pressure and tenderness over the affected sinus	*Streptococcus pneumoniae, Haemophilus influenzae*	NA	NA
Diphtheria	Sore throat, dysphagia, hoarseness, malaise, low-grade fever, tachycardia, nausea, vomiting, chills, and a headache	*Corynebacterium diphtheriae*	NA	NA
Epiglottitis	Fever, sore throat, hoarseness, drooling, dysphagia	*Haemophilus influenzae, Streptococcus pyogenes*	Parainfluenza virus	NA
Flu	Fever, runny nose, sore throat, cough, headache, myalgia, malaise	NA	Influenza virus	NA
Laryngitis	Hoarseness or loss of voice, fever, malaise, dysphagia, sore throat	*Streptococcus pyogenes*	NA	NA
Nasopharyngitis, rhinitis and common cold	Nasal discharge and obstruction; sneezing, sore throat, cough, myalgia, headache	*Streptococcus pyogenes, Haemophilus influenzae*	Rhinovirus	NA
Otitis media	Persistent severe earache accompanied by fever and vomiting. Purulent discharge may be present if tympanic membrane has been perforated	*Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis*	NA	NA
Pertussis (Whooping cough)	Sneezing, runny nose, loss of appetite, listlessness, a hacking cough at night, malaise	*Bordetella pertussis*	NA	NA
Pharyngitis	Red and sore throat, dysphagia, fever, malaise, myalgia, and headache	*Streptococcus pyogenes*	Rhinovirus	*Candida albicans*
Tonsillitis	Red and sore throat, dysphagia, fever, malaise, myalgia, and headache	*Streptococcus pyogenes*	NA	NA

[a] Reference [15]. [b] Reference [16]. [c] Reference [17]. [d] NA=Not applicable.

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Table 2. Pathogens responsible for lower respiratory tract infections.

Infection of the upper respiratory tract	Clinical manifestations[a]	Most common pathogens associated with infection[b,c]		
		Bacterial	Viral	Fungal
Bronchitis	Runny nose, cough, mucopurulent sputum, low-grade fever	*Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*	Rhinovirus, Parainfluenza virus, Influenza virus	*Cryptococcus neoformans*
Pleurisy	Shortness of breath, pain on breathing, pain in the shoulder, dry cough	*Staphylococcus mitis*	NA[d]	NA
Pneumonia	Cough, chest pain, fever, shortness of breath, sputum production, tachycardia, headache, confusion	*Streptococcus pneumoniae*, *Mycoplasma pneumoniae*	Adenovirus, Influenza virus	NA
Legionnaire's disease	Fever, chills, malaise, myalgia, headache, and confusion, cough, difficulty breathing, and breathing may be painful	*Legionella pneumophila*	NA	NA
Tuberculosis	Cough, purulent sputum, cold sweat, with or without fever, malaise, decreased energy and appetite, weight loss, shortness of breath, chest pain	*Mycobacterium tuberculosis*	NA	NA

[a] Reference [15]. [b] Reference [16]. [c] Reference [17]. [d] NA = Not applicable.

Lower respiratory tract infections (LRTI) are defined as acute illnesses, lasting 21 days or less, and are usually associated with a cough and at least one other lower respiratory tract symptoms such as sputum production, dyspnea, wheeze or chest discomfort or pain. Conditions associated with LRTI include, but are not limited to, bronchitis, influenza, pneumonia, Legionnaire's disease and tuberculosis (*Table 2*). Lower respiratory tract infections are often caused by polymicrobial infections, and common pathogens include *Mycoplasma pneumoniae*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Legionella pneumophila* and *Mycobacterium tuberculosis* (*Table 2*). *Mycoplasma pneumoniae* was first identified as having been responsible for causing respiratory related infections in the early 1930s,[27] where it was identified that this pathogen was responsible for a 'primary atypical pneumonia'.[28] *Legionella pneumophila* is responsible for causing Legionnaire's disease, a severe and life-threatening form of pneumonia. This Gram-negative bacillus was first identified in 1977 and since then has been an important cause of community-acquired pneumonia.[28] *Mycobacterium tuberculosis* is a Gram-negative bacillus responsible for causing the highly contagious pulmonary tuberculosis. *M. tuberculosis* is highly virulent and spreads in the air through the diffusion of infected respiratory droplets. Growth of this pathogen within the lungs is associated with extensive damage that ultimately causes death.[29]

Apart from bacterial and viral pathogens, fungal and yeast pathogens also effect the respiratory tract and may result in infections among immunocompromised patients. The most common fungi responsible for pneumonia and other respiratory tract infections include, *Pneumocystis jirovecii*, the *Aspergillus* species, *Candida albicans* and *Cryptococcus neoformans*.[30] Fungal pathogens cause severe and life-threatening fungal pneumonia in immunocompromised hosts due to the lack of cellular protective mechanisms and account for 30.0% of deaths among organ transplant recipients.[31]

The socio-economic impact of these respiratory related infections is substantial with excessive costs linked to treatment, hospitalization and absenteeism. This impact suggests the need for respiratory-related infections to form part of a larger, global primary health priority.[32] According to a World Health Organization (WHO) report released on the prevention and control of chronic respiratory infections, this was considered a priority. The WHO emphasized the need to reduce the mortality and morbidity effects of these infections. The primary objective was aimed at

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strengthening healthcare by identifying new and cost-effective alternatives for treatment.[16] Developments in respiratory infection control have resulted in reduced burden of disease and death over the past two decades, however, The Forum of International Respiratory Societies (FIRS) have more recently reported that as of 2013, four-million deaths were globally attributed to acute infections of the respiratory tract with these infections considered the leading overall cause of death in developing countries. Years following the WHO report on the prevention and control of chronic respiratory infections, these conditions are still the greatest single contributor to the overall burden of disease in the world,[33] further highlighting the need to identify newer, more cost-effective methods for respiratory infection control and treatment.

Current Treatment Regimens

Antibiotics, although effectively used to treat bacterial infections, have contributed to the emergence and spread of antibiotic-resistant bacteria in communities.[4] Resistance to antibiotics is common. A growing level of resistance to β-lactam antibiotics, such as amoxicillin and amoxicillin-clavulanic acid, are on the rise, however, these agents still remain the first line therapy in the management of bacterial respiratory tract infections in both children and adults.[34,35] Antibiotics suggested for the management of respiratory tract infections include, fluoroquinolones (such as erythromycin), macrolide antibiotics (such as clindamycin) and cephalosporins (such as cefpodoxime, cefuroxime, cefdinir and ceftriaxone).

Natural Product Therapy

In recognition of the plight of antibiotic resistance and the allopathic management principles for the treatment of respiratory related infections, a surge in interest in CAM therapies has emerged with 60–80% of developing countries identifying CAM as the most prevalent sources of medicine worldwide.[36] In order to address the effects of growing antimicrobial resistance and increased CAM use, many researchers have focused investigations and occupied their efforts in alignment with natural products such as essential oils.[37] The inhalation of essential oils to cause a desired therapeutic effect is known as aromatherapy, 'aroma' associated to smell and 'therapy' associated to treatment.[38]

Essential oils are applied through topical, oral and inhalation practices for the treatment of respiratory tract infections. Topical application includes the massage or application of diluted essential oils to the surface of the skin. For the treatment of respiratory related infections, it is suggested that essential oils be applied to the chest, back and soles of the feet. Application to the chest and back are indicated for the drainage of sinuses and loosening of phlegm due to the aroma effect of the essential oils applied, while application to the soles of the feet are indicated for the absorption of the essential oil into the bloodstream for a pharmacological effect. Essential oils applied topically have poor absorption with only 5.0% of the applied essential oil transported into the bloodstream.[39]

The second route of essential oil administration is through the ingestion of essential oils. Essential oils are usually formulated by encapsulation in gelatin capsules or diluted in water for oral administration. Bioavailability of essential oils is indicated at approximately 95.0% through oral ingestion, however, the consumption of these essential oils is levelled with a degree of uncertainty regarding safety and as such, not strongly advocated.

The preferred means of essential oil administration for the treatment of respiratory tract infections is through inhalation. Essential oils then distribute to the lungs, with an effective bioavailability of 50.0% (*Figure 1*).

Essential oils, once inhaled, can reach the tiniest spaces within the lungs and due to their effects on mucociliary clearance, are able to remain in these spaces for prolonged periods.[40] Inhalation of essential

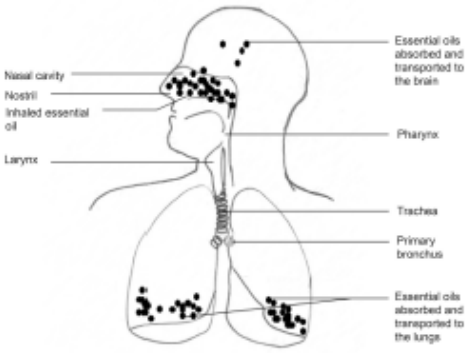


Figure 1. A diagrammatic representation of the route of absorption followed by essential oils once inhaled.

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oils is believed to cause behavior and mood changes thereby treating the patient holistically. Approximately 5.0% of essential oils absorbed in this way are transported to the brain by means of olfactory receptors. Once absorbed through the bloodstream, the essential oils are transported to the liver and kidneys for metabolism and excretion with 70–90.0% of the essential oils absorbed and removed in this way.

Commercially available aroma-therapeutic texts note extensively the use of essential oils for the treatment of respiratory infections, with numerous text sources available for quick reference for treatment regimens. Within the literature investigated,[7,38,41–45] 55 different essential oils were identified for the treatment of respiratory related infections (*Table 3*). An overview of the respiratory uses of essential oils can be seen in *Figure 2*.

Essential oils are mostly used for the treatment of infections caused by bacteria or viruses (total 79.0%). This is followed using these agents in the treatment and management of symptoms most often associated with respiratory infection including excess mucous production, pain and inflammation as well as coughs.

Antimicrobial Susceptibility of Essential Oils

The antimicrobial susceptibility of essential oils has been studied extensively with well over 2'000 publications on the use of these agents for antimicrobial purposes published in the year 2019, alone. A number of antimicrobial susceptibility methods are available for the determination of activity, with varying degrees of reliability.[47,48] Variations in the applied methods for antimicrobial susceptibility testing have implications on the perceived result, as factors such as essential oil extraction method, volume of inoculum, growth phase, culture medium used, pH of media, volatility of essential oil and incubation periods influence results.[49] The most commonly employed methods for antimicrobial susceptibility testing are the minimum inhibitory concentration (MIC) through broth dilution, and disk diffusion (*Figure 3*). The use of disk diffusion assays for antimicrobial testing of essential oils is still widely applied and published; however, this methodology has been fraught with fault and has been deemed by researchers to be an unreliable method for antimicrobial susceptibility testing of essential oils.[50]

Other assays such as the time-kill or death kinetic activity as it is also known as have also been included for review. Time-kill assays indicate the killing effects of a tested sample or combinations of samples dependent on the concentration of each, over time. The methodology includes exposing a test pathogen to a single test sample or a combination of samples at varying concentrations. At predetermined intervals, aliquots of the tested medium are collected and serially diluted. These dilutions are then plated on agar and incubated for a period dependent on the optimum culture conditions of the micro-organism investigated. After incubation, the agar plates are analyzed for growth after exposure to essential oil by counting the colony forming units of test organism survived.[51]

The MIC method of broth dilution for analysis of the antimicrobial activity of essential oils is considered the preferred means of analysis for essential oils due to its increased level of accuracy.[52] For the purposes of this review, only those studies having made use of MIC broth dilution and time-kill methodology have been considered.

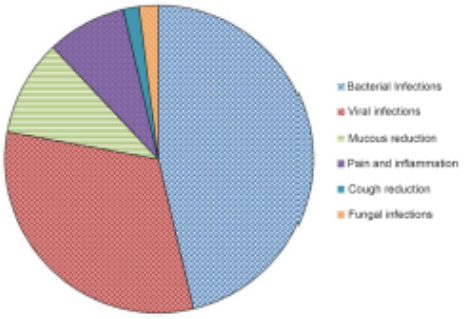


Figure 2. Summary of categorized respiratory conditions in which essential oils are used.

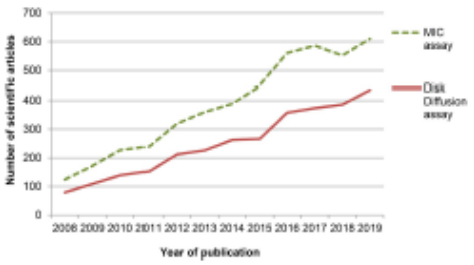


Figure 3. Scientific articles published (PubMed, ScienceDirect, Google Scholar and Scopus), per year on the antimicrobial susceptibility of essential oils comparing methods of the minimum inhibitory concentration with disk diffusion assays.

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Table 3. Essential oils indicated in the treatment of respiratory tract infections.

Essential oil Scientific name	Common name	Respiratory use	References
Abies balsamea	Fir	Chest infections, bronchitis, colds and flu	[41]
Allium sativum	Garlic	Influenza, laryngitis, cough, bronchial catarrh, tuberculosis (TB), diphtheria, chronic bronchitis and whooping cough	[41,45]
Amyris balsamifera	Amyris	Antiseptic, coughs and colds	[41]
Boswellia carterii	Frankincense	Decongestant, asthma, catarrh, colds and a palliative for coughs, bronchitis and laryngitis	[41]
Canarium luzonicum	Elemi	Catarrh, decongestant, bronchitis, cough	[41]
Carum carvi	Caraway	Expectorant, bronchitis, bronchial asthma, laryngitis	[41]
Cedrus atlantica	Cedarwood	Expectorant, bronchitis, coughs and catarrh	[41]
Cinnamomum camphora	Camphor	Decongestant	[41]
Cinnamomum zeylanicum	Cinnamon	Colds, influenza	[41]
Citrus aurantifolia	Lime	Cough, decongestant, catarrh, sinusitis	[41,44]
Citrus sinensis	Orange	Tonsillitis, bronchitis and TB	[41,44]
Citrus bergamia	Bergamot	Antiseptic, sore throats, cold, cough, influenza	[41,46]
Citrus limon	Lemon	Cold, bronchitis	[41,46]
Commiphora myrrha	Myrrh	Expectorant, decongestant, bronchitis, colds, sore throat, catarrh, pharyngitis and coughs	[41,46]
Coriandrum sativum	Coriander	Influenza, chest infections	[41,43,45]
Cupressus sempervirens	Cypress	Antispasmodic, cough, influenza, bronchitis, whooping cough and asthma, astringent	[41,42]
Cymbopogon citratus	Lemon grass	Sore throat, laryngitis	[41,46]
Cymbopogon martinii	Palmarosa	Influenza	[41,43,45]
Daucus carota	Carrot seed	Influenza, bronchitis, cough	[41]
Elettaria cardamomum	Cardamom	Antiseptic	[41,43]
Eucalyptus globulus	Eucalyptus	Antiviral, soothes inflammation, decongestant, influenza, throat infection, cough, catarrh, sinusitis, asthma and TB	[41]
Ferula galbaniflua	Galbanum	Persistent infections of the lungs, decongestant, cough	[41]
Foeniculum vulgare	Fennel	Antiseptic	[41,44]
Helichrysum angustifolium	Immortelle	Cold, influenza, bronchitis, cough, asthma, decongestant	[41]
Hyssopus officinalis	Hyssop	Antiviral, colds, sore throat, influenza, bronchitis, asthma, decongestant	[41,42,44]
Illicium verum	Star anise	Cough	[41,43,45]
Jasminum officinalis	Jasmine	Colds, cough, laryngitis, pharyngitis, bronchitis, pneumonia	[41,43,45]
Laurus nobilis	Bay	Antiseptic, expectorant, bronchitis	[41]
Lavandula angustifolia	Lavender	Bronchitis, asthma, catarrh, cold, laryngitis and throat infection, TB	[41,46]
Lavandula burnati	Lavandin	Bronchitis, throat infections, expectorant, coughs, colds and flu	[41]
Lavandula latifolia cineolifera	Lavender spike	Bronchitis, laryngitis, throat infections, decongestant	[41]
Litsea cubeba	May Chang	Oral thrush	[41,43,45]
Melaleuca alternifolia	Tea tree	Influenza, catarrh	[41,46]
Melaleuca cajuputi	Cajeput	Antiseptic, colds, pharyngitis, laryngitis, and bronchitis, chronic pulmonary disease, asthma	[41,44,46]
Melaleuca viridiflora	Niaouli	Bronchitis, TB, influenza, pneumonia, whooping cough, asthma, sinusitis, catarrh, laryngitis	[41,44]

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Table 3. (cont.)

Essential oil Scientific name	Common name	Respiratory use	References
Mentha pipenta	Peppermint	Bronchitis, pneumonia, TB, influenza	[41,46]
Myrtus communis	Myrtle	Antiseptic, expectorant, throat infections	[41]
Ocimum basilicum	Basil	Antiseptic, influenza, bronchitis, chest infection, whooping cough, sinus congestion, asthma	[41,44]
Origanum majorana	Majoram	Cold, sinusitis, bronchitis, asthma	[41,44,46]
Origanum vulgare	Origanum	Bronchitis, colds and flu, whooping cough	[41]
Pimenta officinalis	Pimento	Chest infections, bronchitis, colds and flu	[41]
Pimpinella anisum	Aniseed	Colds	[41,43,45]
Pinus sylvestris	Pine	Antiseptic, bronchitis, laryngitis, influenza, expectorant	[41,46]
Piper nigrum	Black pepper	Catarrh and colds	[41,43]
Rosa × damascena	Rose	Sore throat, cough	[41,46]
Rosmarinus officinalis	Rosemary	Cold, asthma, chronic bronchitis, influenza	[41,46]
Salvia officinalis	Sage	Cold, catarrh, bronchitis	[41,46]
Salvia sclarea	Clary sage	Asthma, sore throats	[41]
Santalum album	Sandalwood	Sore throats, cough, bronchitis	[41,46]
Styrax benzoin	Benzoin	Bronchitis, asthma, coughs and colds, laryngitis, sore throats, decongestant	[41]
Syzygium aromaticum	Clove	TB, asthma, bronchitis	[41,43,45]
Tagetes patula	Tagetes	Oral thrush	[41,43,45]
Thymus vulgaris	Thyme	Cold, cough, sore throats, tonsillitis, laryngitis, pharyngitis, bronchitis, whooping cough, asthma	[41,46]
Viola odorata	Violet	Diphtheria, laryngitis, decongestant	[41,43,45]
Zingiber officinale	Ginger	Antiseptic, influenza, throat infections, colds, antiseptic	[41,44]

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The Antibacterial Activity of Essential Oils

Antibacterial Mechanism of Action of Essential Oils

Essential oils demonstrate varying complex mechanisms of antimicrobial effects dependent on the chemistry within the essential oil. Essential oils are often comprised of several different classes of chemical constituents, each of which may prove to have a differing antimicrobial effect. In addition, the antibacterial activity of essential oils also differs based on the architectural structure of the bacterial pathogen. According to Swamy et al.,[53] essential oils primarily destabilize the cellular structure of bacterial pathogens, leading to the breakdown of membrane integrity. The antimicrobial potential of essential oils varies among Gram-positive and Gram-negative bacteria due to the variations in density of the bacterial cell wall. Essential oils are lipophilic in nature and as such, are taken up quickly within Gram-positive bacterium due to their dense lipophilic phospholipid bilayer within cell walls. Essential oils therefore elicit a prompt antimicrobial effect at the junction of the cell wall as well as within the cell cytoplasm.[54] Damage to the cell membrane results in increased permeability of the cell preventing cellular activities, such as energy production, membrane transport, metabolic regulatory functions and increased leakage of cellular components and loss of ions.[53,55] Within the cell cytoplasm, essential oils have demonstrated the ability to disrupt proton pumps and deplete intracellular-ATP. These events all cascade to cause cytoplasmic coagulation of inner cellular components which cause death to the cell.[56]

Gram-Positive Bacteria

Pneumococcal infections, including *Streptococcal* species, have been identified by the WHO as causing the greatest number of respiratory infections among Gram-positive bacteria.[16] Pneumococcal infections are generally associated with initial colonization of these bacteria within the nasopharynx. Due to the anatomy of the nasopharynx, steady progression of infection occurs with colonization of pneumococcal pathogens within the lower parts of the respiratory tract, eventually leading to systemic disease.[57,58] Pneumococcal infections are readily spread among susceptible individuals, such as children and the elderly, through droplet formation and transmission from the nasopharynx colonization through sneezing and coughing.[59] *Table 4* shows some of the antimicrobial *in vitro* studies undertaken on commercial essential oils against Gram-positive pathogens responsible for respiratory related infections.

Of the 55 available commercial essential oils documented within the aroma-therapeutic literature for use in respiratory infections, only a minority have been tested against respiratory related, Gram-positive pathogens by means of broth dilution, of which the majority have been against the micro-organism *Staphylococcus aureus*. The pathogen, *S. aureus*, is not unique to respiratory infections and is responsible for a great number of other infections including the skin and other body systems resulting in impetigo, meningitis and septicemia.[84] When compared to the WHO classification of the pathogens most responsible for respiratory infections (*Tables 1* and *2*), the literature shows a great disparity in available data on the antimicrobial potential of essential oils, specifically for respiratory infections caused by Gram-positive bacteria (*Figure 4*).

Figure 4 indicates that *S. pyogenes*, the pathogen most responsible for infections of the upper and lower respiratory tract (largest circle), has far less scientific data published on the effects of essential oils against it when compared to the pathogen, *S. aureus*, with limited pathogenesis for respiratory infections (smallest circle). This result is augmented further by the disparity in prominence for respiratory infections caused by *S. pneumoniae* and the availability of scientific data to validate the aroma-therapeutic claims for essential oil use in treating infections caused by this micro-organism. This outcome is of great concern

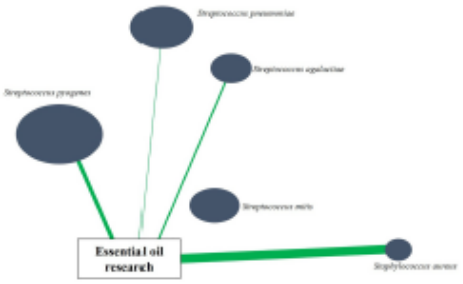


Figure 4. Activity map indicating the quantity of scientific publications (connector bar) reporting on respiratory Gram-positive pathogens compared to the prevalence of these micro-organism to cause respiratory infection (circle). The thicker the connector bar, the greater the number of publications available for essential oil use against this pathogen. The larger the circle, the greater the prevalence for respiratory pathogenesis as determined by the WHO.

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Table 4. Some essential oil studies showing efficacy against Gram-positive pathogens associated with respiratory tract infections.

Essential oil Scientific name	Common name	Pathogen strain	Result (MIC)	Main chemical constituent	Reference
Staphylococcus aureus					
Cinnamomum zeylanicum	Cinnamon	ATCC 25923	3.2 mg/ml	Cinnamaldehyde (52.42%)	[60]
		ATCC 25923	1.0 mg/ml	Cinnamaldehyde (92.4%)	[61]
		ATCC 29213	1.25 µl/ml	n.d.[a]	[62]
		clinical isolate	0.0125 ml/ml	n.d.	[63]
Citrus aurantium	Lime	ATCC 25923	12.8 mg/ml	n.d.	[60]
Citrus limon	Lemon	ATCC 25923	> 12.8 mg/ml	n.d.	[60]
		ATCC 6538	6.67 mg/ml	n.d.	[64]
Citrus sinensis	Orange	ATCC 25923	> 12.8 mg/ml	n.d.	[60]
Coriandrum sativum	Coriander	ATCC 25923	108.0 mg/ml	Decanal (14.3%)	[65]
Eucalyptus globulus	Eucalyptus	ATCC 25923	10.0 mg/ml	1,8-Cineole (81.9%)	[66]
Eugenia caryophyllus	Clove	ATCC 25923	> 6.4 mg/ml	n.d.	[60]
		ATCC 29213	2.5 µl/ml	n.d.	[62]
		clinical isolate	5.0 µl/ml	n.d.	[62]
Juniperus communis	Juniper	ATCC 25923	10.0 mg/ml	α-Pinene (39.8%)	[67]
Lavandula angustifolia	Lavender	ATCC 25923	1.15 mg/ml	Linalool (40.3%)	[68]
		ATCC 25923	2.0 mg/ml	Linalool (57.1%)	[69]
Lavandula intermedia	Lavandin	ATCC 25923	1.5 mg/ml	Linalool (54.0%)	[69]
Melaleuca alternifolia	Tea tree	ATCC 25923	6.7 mg/ml	n.d.	[70]
Mentha piperita	Peppermint	ATCC 6538	0.60 mg/ml	Menthol (47.3%)	[67]
		ATCC 6538	0.63 mg/ml	Menthol (47.5%)	[68]
		ATCC 25923	8.0 mg/ml	n.d.	[70]
		ATCC 6538	0.625 mg/ml	Menthone (27.9%)	[71]
		ATCC 6538	0.625 mg/ml	Menthol (42.3%)	[71]
		ATCC 6538	1.25 mg/ml	Menthol (39.9%)	[71]
		ATCC 6538	2.50 mg/ml	Menthol (34.6%)	[71]
Myrtus communis	Myrtle	CIP 7625	20.0 µl/ml	α-Pinene (44.62%)	[72]
Origanum vulgare	Origanum	clinical isolate	0.36 mg/ml	Carvacrol (65.93%)	[73]
		clinical isolate	0.10 mg/ml	γ-Terpinene (20.5%)	[74]
		ATCC 33591	0.50 mg/ml	n.d.	[75]
Pelargonium graveolens	Geranium	ATCC 25923	> 12.8 mg/ml	n.d.	[60]
Pinus sylvestris	Pine	clinical isolate	2.5 mg/ml	α-Pinene (41.90%)	[76]
			125 mg/ml	α-Pinene (41.90%)	[70]
Rosmarinus officinalis	Rosemary	ATCC 25923	> 12.8 mg/ml	n.d.	[60]
		ATCC 25923	6.0 mg/ml	n.d.	[70]
Salvia officinalis	Sage	clinical isolate	7.49 mg/ml	n.d.	[77]
Syzygium aromaticum	Clove		0.0125 ml/ml	n.d.	[62]
Thymus vulgaris	Thyme	ATCC 25923	4.7 mg/ml	n.d.	[70]
		clinical isolate	0.02 mg/ml	n.d.	[78]
		clinical isolate	0.0125 ml/ml	n.d.	[63]
Zingiber officinalis	Ginger (leaf)	ATCC 12600	0.16 mg/ml	β-Caryophyllene (31.7%)	[79]

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Table 4. (cont.)

Essential oil Scientific name	Common name	Pathogen strain	Result (MIC)	Main chemical constituent	Reference
	Ginger (rhizome)	ATCC 12600	0.31 mg/ml	Camphene (14.5%)	[79]
Streptococcus agalactiae					
Cinnamomum zeylanicum	Cinnamon	clinical isolate	0.0125 ml/ml	n.d.	[63]
Eucalyptus globulus	Eucalyptus	ATCC 27956	20.0 mg/ml	1,8-Cineole (81.9%)	[67]
Juniperus communis	Juniper berry	clinical isolate	20.0 mg/ml	α-Pinene (39.8%)	[67]
Mentha piperita	Peppermint	clinical isolate	2.5 mg/ml	Menthol (47.3%)	[67]
Syzygium aromaticum	Clove	clinical isolate	0.0125 ml/ml	n.d.	[63]
Thymus vulgaris	Thyme	clinical isolate	6.25 µl/ml	n.d.	[63]
Streptococcus pyogenes					
Cinnamomum zeylanicum	Cinnamon	clinical isolate	6.25 µl/ml	n.d.	[63]
		clinical isolate	0.41 mg/ml	*trans*-Cinnamaldehyde (45.9%)	[80]
Cymbopogon citratus	Lemongrass	CIP 104226	0.93% (v/v)	Geranial (43.4%)	[81]
Eucalyptus globulus	Eucalyptus	ATCC 12344	10.00 mg/ml	1,8-Cineole (81.9%)	[67]
		clinical isolate	2.82 mg/ml	1,8-Cineole (91.0%)	[80]
Eucalyptus odorata	Eucalyptus	clinical isolate	9.79 mg/ml	Cryptone (20.9%)	[82]
Juniperus communis	Juniper berry	ATCC 12344	20.00 mg/ml	α-Pinene (39.8%)	[67]
Lavandula angustifolia	Lavender	clinical isolate	1.20 mg/ml	Linalool (40.3%)	[68]
		ATCC 12204	1.0 mg/ml	Linalool (57.1%)	[69]
Lavandula intermedia	Lavandin	ATCC 12204	1.0 mg/ml	Linalool (54.0%)	[69]
Melaleuca leucadendra	Cajuput	ATCC 12344	5.00 mg/ml	1,8-Cineole (67.6%)	[67]
Mentha piperita	Peppermint	ATCC 12344	5.00 mg/ml	Menthol (47.3%)	[67]
		clinical isolate	0.35 mg/ml	Menthol (27.2%)	[80]
		clinical isolate	0.63 mg/ml	Menthol (47.5%)	[68]
Pinus sylvestris	Pine	clinical isolate	1.35 mg/ml	α-Pinene (26.1%)	[80]
Syzygium aromaticum	Clove	clinical isolate	0.0125 ml/ml	n.d.	[63]
		clinical isolate	0.10 mg/ml	Eugenol (66.9%)	[80]
Thymus vulgaris	Thyme	clinical isolate	0.0125 ml/ml	n.d.	[63]
		clinical isolate	0.43 mg/ml	Thymol (46.1%)	[80]
Streptococcus pneumoniae					
Cinnamomum zeylanicum	Cinnamon	clinical isolate	0.00625 ml/ml	n.d.	[63]
		DSM 20566	0.06 mg/ml	*trans*-Cinnamaldehyde (45.9%)	[80]
Citrus hystrix	Makrut lime (fruit)	clinical isolate	0.30 mg/ml	Limonene (40.7%)	[83]
	Makrut lime (leaf)	clinical isolate	0.30 mg/ml	Citronellal (80.0%)	[83]
Eucalyptus globulus	Eucalyptus	clinical isolate	1.41 mg/ml	1,8-Cineole (91.0%)	[80]
Mentha piperita	Peppermint	clinical isolate	0.35 mg/ml	Menthol (27.2%)	[80]
Pinus sylvestris	Pine	clinical isolate	0.68 mg/ml	α-Pinene (26.1%)	[80]
Syzygium aromaticum	Clove	clinical isolate	0.25 mg/ml	Eugenol (66.9%)	[80]
		clinical isolate	0.0125 ml/ml	n.d.	[63]

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Table 4. (cont.)

Essential oil Scientific name	Common name	Pathogen strain	Result (MIC)	Main chemical constituent	Reference
Thymus vulgaris	Thyme	clinical isolate	0.00625 ml/ml	n.d.	[63]
		DSM 20566	0.11 mg/ml	Thymol (46.1%)	[80]
Streptococcus species					
Citrus hystrix	Makrut lime (fruit)	*Group A Strep* clinical isolate	0.30 mg/ml	Limonene (40.7%)	[83]
	Makrut lime (leaf)	*Group A Strep* clinical isolate	0.30 mg/ml	Citronellal (80.0%)	[83]
	Makrut lime (fruit)	*Group B Strep* clinical isolate	0.30 mg/ml	Limonene (40.7%)	[83]
	Makrut lime (leaf)	*Group B Strep* clinical isolate	0.30 mg/ml	Citronellal (80.0%)	[83]
	Makrut lime (fruit)	*Group C Strep* clinical isolate	0.30 mg/ml	Limonene (40.7%)	[83]
	Makrut lime (leaf)	*Group F Strep* clinical isolate	0.30 mg/ml	Citronellal (80.0%)	[83]
Citrus hystrix	Makrut lime (fruit)	*Group F Strep* clinical isolate	0.50 mg/ml	Limonene (40.7%)	[83]
	Makrut lime (fruit)	*Group G Strep* clinical isolate	0.30 mg/ml	Limonene (40.7%)	[83]
	Makrut lime (leaf)	*Group G Strep* clinical isolate	0.30 mg/ml	Citronellal (80.0%)	[83]

[a] Not determined.

as essential oils are extensively cited within aromatherapeutic literature for providing widespread antimicrobial potential when inhaled or ingested for respiratory related infections and symptoms, however, from the data obtained, it can be determined that these statements are largely unfounded when compared to evidence against the most prominent pathogens associated with respiratory infections.

From the literature reviewed, it was determined that essential oils have moderate potential for antimicrobial effects against the *Streptococcal* pathogens commonly associated with respiratory infections. Noteworthy antimicrobial activity is considered for essential oils exhibiting MIC values $\leq$ 1.00 mg/ml, while moderate antimicrobial activity is classified for MIC values $>$ 1.00 mg/ml and $<$ 5.00 mg/ml.[50] Essential oils with MIC values of more than or equivalent to 5.00 mg/ml are considered as having little antimicrobial effects. With this in mind, studies,[63,67] against the pathogen *S. agalactiae* demonstrated that only one sample (*Mentha piperita* with an MIC value of 2.50 mg/ml) of the six essential oils investigated displayed moderate antimicrobial activity, while the other five essential oils (*Cinnamomum zeylanicum* (cinnamon), *Eucalyptus globulus* (eucalyptus), *Juniperus communis* (juniper berry), *Syzygium aromaticum* (clove) and *Thymus vulgaris* (thyme)) proved to have no antimicrobial potential with MIC values of greater than 5.00 mg/ml. Against the pathogen, *S. pyogenes*, *M. piperita* confirmed antimicrobial potential with a noteworthy MIC value of 0.63 mg/ml.[68] *Mentha piperita* is indicated within aroma-therapeutic texts[7,38,41–45] for the management of infections associated to the lower respiratory tract including, bronchitis, pneumonia, tuberculosis (TB) and influenza, however, *S. pyogenes* is a pathogen most commonly associated with upper respiratory tract infections including sinusitis, laryngitis and pharyngitis. The essential oil of lavender, *Lavandula angustifolia*, proved to have moderate antimicrobial potential against the pathogen *S. pyogenes* with an MIC value of 1.20 mg/ml.[68] *Lavandula angustifolia* is indicated within aroma-therapeutic texts for the management of both upper and lower respiratory infections such as bronchitis and laryngitis.[7,38,41–45] The essential oil most effective against this pathogen, was noted as *Syzygium aromaticum* with an MIC of 0.10 mg/ml.[80] *Syzygium aromaticum*, as with *L. angustifolia* and *M. piperita* is associated with the management of conditions of the lower respiratory tract and include, TB, asthma and bronchitis.[7,38,41–45] Noteworthy antimicrobial effects were determined against the lower respiratory tract infection causing pathogen, *S.*

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pneumoniae,[63,80] with the essential oil *Cinnamomum zeylanicum* determined the most effective in antimicrobial activity with an MIC of 0.06 mg/ml.[80] *Cinnamomum zeylanicum* is indicated within aroma-therapeutic texts for the management of infections associated to the common cold and influenza.[7,38,41–45]

Gram-Negative Bacteria

Gram-negative bacteria possess a more complex cell wall when compared with Gram-positive bacteria, making them more resistant to the antimicrobial effects of essential oils. The porin channels responsible for transmission of external substances into the cell of Gram-negative bacteria are resistant to hydrophobic compounds and as such, essential oils are taken up more slowly into the cells of these pathogens. The antimicrobial effect is thus hindered, and the bacteria are more resistant to essential oils.[85]

Haemophilus influenzae is the pathogen most commonly isolated from respiratory infections of the upper and lower respiratory tract according to the WHO classification of infection (*Tables 1* and *2*). *H. influenzae*, as with pneumococcal species, initially colonizes the nasopharynx. Initiation of infection results due to the disruption in host immunity and spreads both systemically and through the respiratory tract, causing widespread infection.[86] *H. influenzae* is characterized into two forms, either typeable or non-typeable, based on the presence or absence of the polysaccharide capsule. The polysaccharide capsule aids in assisting the pathogen to avoid detection and immune responses such as phagocytosis, allowing non-typeable forms of *H. influenzae* to cause respiratory related infections.[86] This pathogen has evolved in its attempt to evade host detection and maintain host infection, with varying classes of strains developing annually due to resistance.

Table 5 shows the antimicrobial *in vitro* studies undertaken on commercial essential oils against Gram-negative pathogens responsible for respiratory related infections by means of broth dilution assays.

Of the 55 available commercial essential oils documented within the aroma-therapeutic literature for use in respiratory infections, 25 oils have been tested against respiratory related Gram-negative pathogens by means of broth dilution. When compared to the WHO classification of the most responsible pathogens for respiratory infections (*Tables 1* and *2*), the literature sourced once again shows a great disparity in available research on the Gram-negative bacteria most commonly isolated within respiratory tract infections (*Figure 5*).

The pathogen *Klebsiella pneumoniae* is the most studied respiratory pathogen (*Figure 5*), yet, the pathogen most commonly associated with infection, *H. influenzae*, has far less scientific data available on validating essential oil efficacy. This is further highlighted against the micro-organism *Moraxella catarrhalis*, commonly associated with lower respiratory tract infections, yet little related research could be identified. *Klebsiella pneumoniae* is an uncommon pathogen responsible for nosocomial pneumonia infections in immunocompromised hosts and has more than 80 identifiable serotypes, most of which cause acute and severe infection at onset with a mortality rate as high as 58.0%.[95,96] Serotypes of *K. pneumoniae* are also identified as having a tough outer polysaccharide capsule that allows the pathogen to go undetected. Treatment of infections caused by this pathogen is extensive with both oral and intravenous antibiotics administered for periods of two weeks or longer.[95,97] The antimicrobial potential of essential oils against this pathogen is promising with several oils presenting with noteworthy antimicrobial effects (*Table 5*). From the literature sourced, 61.0% of essential oils investigated demonstrated noteworthy to moderate antimicrobial effect. The essential oils with the greatest effect were *Foeniculum vulgare* (fennel), *Mentha piperita*, *Ocimum basilicum* (basil), *Origanum majorana* (marjoram) and *Origanum vulgare* (origanum) with MIC values of 0.01 mg/ml.[89] These essential oils are noted within aroma-therapeutic texts for their effect on

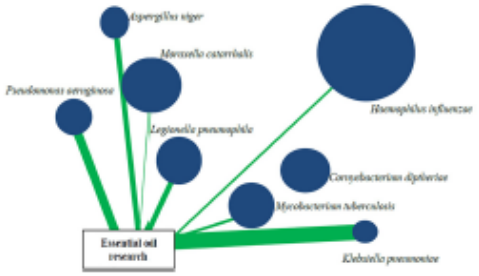


Figure 5. Activity map indicating the quantity of scientific publications (connector bar) reporting on respiratory Gram-negative pathogens compared to the prevalence of these micro-organism to cause respiratory infection (circle). The thicker the connector bar, the greater the number of publications available for essential oil use against this pathogen. The larger the circle, the greater the prevalence for respiratory pathogenesis as determined by the WHO.

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Table 5. Some essential oil studies showing efficacy against Gram-negative pathogens associated with respiratory tract infections.

Essential oil Scientific name	Common name	Pathogen strain	Result (MIC)	Main chemical constituent	Reference
Pseudomonas aeruginosa					
Cinnamomum zeylanicum	Cinnamon	ATCC 27853	>0.8 mg/ml	Cinnamaldehyde (52.4%)	[60]
Citrus aurantium	Lime	ATCC 27853	>6.4 mg/ml	n.d.[a]	[60]
Citrus limon	Lemon	ATCC 27853	12.8 mg/ml	n.d.	[60]
Citrus sinensis	Orange	ATCC 10145	>12.8 mg/ml	n.d.	[60]
Eucalyptus globulus	Eucalyptus	ATCC 27853	0.02 mg/ml	n.d.	[87]
		ATCC 27853	10.0 mg/ml	1,8-Cineole (81.9 %)	[67]
		ATCC 27853	32.0 µl/ml	1,8-Cineole (63.81 %)	[66]
		clinical isolates	0.10 mg/ml	n.d.	[87]
		PA 08	32.0 µl/ml	1,8-Cineole (63.81 %)	[66]
		PA 12/08	32.0 µl/ml	1,8-Cineole (63.81 %)	[66]
Eugenia caryophyllus	Clove	ATCC 27853	>1.6 mg/ml	n.d.	[60]
Juniperus communis	Juniper berry	ATCC 27853	20.0 mg/ml	α-Pinene (39.8 %)	[67]
Lavandula angustifolia	Lavender	clinical isolate	2.33 mg/ml	Linalool (40.3 %)	[68]
Melaleuca leucadendra	Cajuput	ATCC 27853	5.0 mg/ml	1,8-Cineole (67.6 %)	[67]
Mentha piperita	Peppermint	ATCC 27853	20.0 mg/ml	Menthol (47.3 %)	[67]
		ATCC 27853	2.5 mg/ml	Menthone (27.9 %)	[71]
		ATCC 27853	2.5 mg/ml	Menthol (42.3 %)	[71]
		ATCC 27853	5.0 mg/ml	Menthol (39.9 %)	[71]
		ATCC 27853	2.5 mg/ml	Menthol (34.6 %)	[71]
		clinical isolate	1.13 mg/ml	Menthol (47.5 %)	[68]
Ocimum gratissimum	Basil	ATCC 15442	≥1.02 mg/ml	Eugenol (65.3 %)	[88]
		clinical isolate	≥1.02 mg/ml	Eugenol (65.3 %)	[88]
Origanum vulgare	Origanum	clinical isolate	>0.20 mg/ml	γ-Terpinene (20.5 %)	[74]
Pelargonium graveolens	Geranium	ATCC 27853	>12.8 mg/ml	n.d.	[60]
Rosmarinus officinalis	Rosemary	ATCC 27853	>6.4 mg/ml	n.d.	[60]
Salvia officinalis	Sage	867	7.49 mg/ml	n.d.	[77]
Thymus vulgaris	Thyme	clinical isolate	>0.50 mg/ml	n.d.	[78]
Klebsiella pneumoniae					
Cinnamomum zeylanicum	Cinnamon	ATCC 15380	3.2 mg/ml	Cinnamaldelyde (52.4%)	[60]
		clinical isolate	0.01 25 ml/ml	n.d.	[63]
Citrus aurantium	Lime	ATCC 15380	>6.4 mg/ml	n.d.	[60]
Citrus limon	Lemon	ATCC 13883	16.0 mg/ml	n.d.	[64]
		ATCC 15380	>12.8 mg/ml	n.d.	[60]
Citrus sinensis	Orange	ATCC 15380	12.8 mg/ml	n.d.	[60]
Coriandrum sativum	Coriander	clinical isolate	163.0 mg/ml	Decanal (14.3 %)	[65]
Eucalyptus globulus	Eucalyptus	ATCC 13883	16.0 µl/ml	1,8-Cineole (63.81 %)	[66]
		ATCC 700603	20.0 mg/ml	1,8-Cineole (81.9 %)	[67]
		KP 08	32.0 µl/ml	1,8-Cineole (63.81 %)	[66]
Eugenia caryophyllus	Clove	ATCC 15380	>6.4 mg/ml	n.d.	[60]

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Table 5. (cont.)

Essential oil Scientific name	Common name	Pathogen strain	Result (MIC)	Main chemical constituent	Reference
Foeniculum vulgare	Fennel	RSKK 574	0.01 mg/ml	*trans*-Anethol (80.73%)	[89]
Juniperus communis	Juniper berry	ATCC 700603	40.0 mg/ml	α-Pinene (39.8%)	[67]
Lavandula angustifolia	Lavender	ATCC 10031	1.5 mg/ml	Linalool (57.1%)	[69]
Lavandula intermedia	Lavandin	ATCC 10031	1.5 mg/ml	Linalool (54.0%)	[69]
Melaleuca alternifolia	Tea tree	NCTC 9633	6.0 mg/ml	n.d.	[70]
Melaleuca leucadendra	Cajuput	ATCC 700603	10.0 mg/ml	1,8-Cineole (67.6%)	[67]
Mentha piperita	Peppermint	ATCC 700603	5.0 mg/ml	Menthol (47.3%)	[67]
		clinical isolate	2.5 mg/ml	Menthone (27.9%)	[71]
		clinical isolate	2.5 mg/ml	Menthol (42.3%)	[71]
		clinical isolate	2.5 mg/ml	Menthol (39.9%)	[71]
		clinical isolate	2.5 mg/ml	Menthol (34.6%)	[71]
		NCTC 9633	8.0 mg/ml	n.d.	[70]
		RSKK 574	0.01 mg/ml	Isomenthone (50.08%)	[89]
Mentha spicata	Spearmint	RSKK 574	0.01 mg/ml	Carvone (75.07%)	[89]
Myrtus communis	Myrtle	clinical isolate	20.0 µl/ml	α-Pinene (44.62%)	[72]
Ocimum basilicum	Basil	RSKK 574	0.01 mg/ml	Linalool (75.94%)	[89]
Origanum majorana	Marjoram	RSKK 574	0.01 mg/ml	Carvacrol (79.58%)	[89]
Origanum vulgare	Origanum	AFRRI 7	0.50 mg/ml	n.d.	[75]
		RSKK 574	0.01 mg/ml	Carvacrol (81.25%)	[89]
Pelargonium graveolens	Geranium	ATCC 15380	12.8 mg/ml	n.d.	[60]
Pinus sylvestris	Pine	clinical isolate	1.25 mg/ml	α-Pinene (41.9%)	[76]
		clinical isolate	10.0 mg/ml	α-Pinene (41.9%)	[76]
Rosmarinus officinalis	Rosemary	ATCC 15380	>12.8 mg/ml	n.d.	[70]
		NCTC 9633	8.0 mg/ml	n.d.	[70]
Salvia officinalis	Sage	ATCC 1603	14.99 mg/ml	n.d.	[77]
Thymus vulgaris	Thyme	clinical isolate	>0.50 mg/ml	n.d.	[78]
		clinical isolate	0.025 ml/ml	n.d.	[63]
		NCTC 9633	4.0 mg/ml	n.d.	[70]
Zingiber officinalis	Ginger (leaf)	ATCC 13883	0.47 mg/ml	β-Caryophyllene (31.7%)	[79]
	Ginger (rhizome)	ATCC 13883	0.47 mg/ml	Camphene (14.5%)	[79]
Haemophilus influenzae					
Cinnamomum zeylanicum	Cinnamon	clinical isolate	0.00625 ml/ml	n.d.	[63]
		DSM 4690	0.06 mg/ml	*trans*-Cinnamaldehyde (45.9%)	[80]
Citrus hystrix	Makrut lime (fruit)	clinical isolate	0.50 mg/ml	Limonene (40.7%)	[83]
	Makrut lime (leaf)	clinical isolate	0.50 mg/ml	Citronellal (80.0%)	[83]
Eucalyptus globulus	Eucalyptus	clinical isolate	1.41 mg/ml	1,8-Cineole (91.0 %)	[80]
Mentha piperita	Peppermint	clinical isolate	0.21 mg/ml	Menthol (27.2%)	[80]
Pinus sylvestris	Pine	clinical isolate	1.35 mg/ml	α-Pinene (26.1%)	[80]
Syzygium aromaticum	Clove	clinical isolate	0.25 mg/ml	Eugenol (66.9 %)	[80]
	Clove	clinical isolate	0.0125 ml/ml	n.d.	[80]
Thymus vulgaris	Thyme	clinical isolate	0.00625 ml/ml	n.d.	[63]

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Table 5. (cont.)

Essential oil Scientific name	Common name	Pathogen strain	Result (MIC)	Main chemical constituent	Reference
		DSM 4690	0.11 mg/ml	Thymol (46.10 %)	[80]
Legionella pneumophila					
Citrus sinensis	Orange	GC-11	>0.90 mg/ml	*cis*-Linalool oxide (24.9 %)	[90]
		GC-13	0.90 mg/ml	*cis*-Linalool oxide (24.9 %)	[90]
		GC-3	>0.90 mg/ml	*cis*-Linalool oxide (24.9 %)	[90]
		GC-4	0.45 mg/ml	*cis*-Linalool oxide (24.9 %)	[90]
		GC-5	0.11 mg/ml	*cis*-Linalool oxide (24.9 %)	[90]
		GC-6	0.90 mg/ml	*cis*-Linalool oxide (24.9 %)	[90]
Melaleuca alternifolia	Tea tree	ATCC 33152	0.25 % (v/v)	n.d.	[91]
		ATCC 33215	0.25 % (v/v)	n.d.	[91]
		clinical isolate	0.125–0.5 % (v/v)	n.d.	[91]
Rosmarinus officinalis	Rosemary	GC-11	0.55 mg/ml	1,8-Cineole (59.18 %)	[90]
		GC-13	1.10 mg/ml	1,8-Cineole (59.18 %)	[90]
		GC-3	>1.10 mg/ml	1,8-Cineole (59.18 %)	[90]
		GC-4	0.28 mg/ml	1,8-Cineole (59.18 %)	[90]
		GC-5	0.07 mg/ml	1,8-Cineole (59.18 %)	[90]
		GC-6	0.55 mg/ml	1,8-Cineole (59.18 %)	[90]
Thymus vulgaris	Thyme	GC-11	<0.03 mg/ml	Carvacrol (88.50 %)	[90]
		GC-13	0.07 mg/ml	Carvacrol (88.50 %)	[90]
		GC-3	<0.03 mg/ml	Carvacrol (88.50 %)	[90]
		GC-4	<0.03 mg/ml	Carvacrol (88.50 %)	[90]
		GC-5	<0.03 mg/ml	Carvacrol (88.50 %)	[90]
		GC-6	<0.03 mg/ml	Carvacrol (88.50 %)	[90]
Moraxella catarrhalis					
Citrus hystrix	Makrut lime (fruit)	clinical isolate	0.25 mg/ml	Limonene (40.7 %)	[83]
Citrus limon	Lemon	ATCC 23246	8.0 mg/ml	n.d.	[80]
Cinnamomum zeylanicum	Cinnamon	DSM 4690	0.10 mg/ml	*trans*-Cinnamaldelyde (45.9 %)	[80]
Eucalyptus globulus	Eucalyptus	DSM 4690	2.81 mg/ml	1,8-Cineole (91.0 %)	[80]
Mentha piperita	Peppermint	DSM 4690	0.35 mg/ml	Menthol (27.2 %)	[80]
Pinus sylvestris	Pine	DSM 4690	0.34 mg/ml	α-Pinene (26.1 %)	[80]
Syzygium aromaticum	Clove	DSM 4690	0.25 mg/ml	Eugenol (66.9 %)	[80]
Mycobacterium species					
Citrus bergamia	Bergamot	clinical isolate	0.50 % (v/v)	Limonene (33.7 %)	[92]
Citrus limon	Lemon	ATCC 14468	>16.0 mg/ml	n.d.	[93]
Myrtus communis	Myrtle	clinical isolate	0.17 % (v/v)	Limonene (n.m.[b])	[93]
		H37Ra	0.17 % (v/v)	Limonene (n.m.[b])	[93]
		H37Rv	0.17 % (v/v)	Limonene (n.m.[b])	[93]
Zingiber officinale	Ginger	H37Rv (ATCC 27294)	0.62–1.25 mg/ml	α-Zingiberene (16.40 %)	[94]

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Table 5. (cont.)

Essential oil Scientific name	Common name	Pathogen strain	Result (MIC)	Main chemical constituent	Reference
			1.25–2.50 mg/ml	Citronellol (21.20%)	[94]
			> 2.50 mg/ml	*ar*-Curcumene (43.55%)	[94]
			> 2.50 mg/ml	Citronellal (41.66%)	[94]
			0.62–2.50 mg/ml	1,8-Cineole (31.25%)	[94]
			0.31–1.25 mg/ml	γ-Eudesmol (25.20%)	[94]
			0.62–2.50 mg/ml	*cis*-Carveol (30.63%)	[94]
			1.25–2.50 mg/ml	(E)-Nerolidol (43.49%)	[94]
			1.25–2.50 mg/ml	Zingiberenol (37.69%)	[94]
			> 2.50 mg/ml	Geraniol (47.91%)	[94]

[a] Not determined. [b] Value not mentioned.

general chest infections and for the treatment of lower respiratory infections such as bronchitis and pneumonia.[41] Other essential oils such as *Zingiber officinalis* (ginger) also demonstrated noteworthy antimicrobial potential against this pathogen with an MIC value of 0.47 mg/ml.[79] The antimicrobial effect of these essential oils against Gram-negative bacteria have been supported by a number of other studies highlighting the potential of these samples for the treatment of infections caused by Gram-negative pathogens other than *K. pneumoniae*.[98–100]

Legionella pneumophilia is a common cause of pneumonia within immunocompromised hosts and is spread through inhalation of contaminated water source aerosols. The bacteria survive and grow in protozoa of contaminated water sources within natural environments such as dams and lakes as well as within artificial water systems. Transmission is not related to human-to-human exposure.[101] Treatment of *L. pneumophilia* pneumonia requires the use of antibiotics with mortality in untreated patients occurring as high as 40.0–80.0%.[101] Of the essential oils investigated against this pathogen, the vast majority (72.0%) have been identified as having noteworthy antimicrobial potential with MIC values < 1.00 mg/ml.[90] Other studies have identified moderate antimicrobial activity with effects ranging from 2.25 mg/ml to 4.50 mg/ml.[91] The antimicrobial study of essential oils against *L. pneumophilia* is not extensive due to the hazardous materials associated with the growth medium used for laboratory testing. Specialized equipment and facilities associated with the study of *L. pneumophilia* limits further investigations.

The essential oil with the greatest antimicrobial potential against this pathogen was identified as *T. vulgaris* of which an MIC value of < 0.03 mg/ml was observed against four varying strains of *L. pneumophilia*.[90] Thyme essential oil is recorded to be effective against respiratory pathogens of the upper and lower respiratory tract.[41] It should be noted, however, that *T. vulgaris* has been proven to be toxic, and as such has limited potential for internal or oral use.

Moraxella catarrhalis, formerly known as *Neisseria catarrhalis* or *Micrococcus catarrhalis*, is a commonly identified commensal of the upper respiratory tract, with a particular preferential attachment to the oropharyngeal epithelial cells.[102] *Moraxella catarrhalis* has been identified as a significant cause of upper and lower respiratory tract infections in healthy children and elderly patients and a well-known cause for pneumonia-mortality in immunocompromised patients.[103] Virulence of *M. catar-*

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rhalis is characterized by the tough polysaccharide capsule enveloping this micro-organism with increased levels of resistance developing against first-line treatments such as penicillin.[103,104] Only three studies could be identified on the investigation of essential oils against this micro-organism using broth dilution assays.[80,83,105] The essential oils of *Citrus limon* and *C. hystrix* (makrut lime) were investigated for their antimicrobial effect against *M. catarrhalis* with MIC values of 8.0 mg/ml (not active) and 0.25 mg/ml identified, respectively.[80,83,105]. *C. hystrix*, or makrut lime, essential oil is a native citrus to Asia and has been shown to elicit excellent antimicrobial activity against Gram-negative pathogens with MIC values ranging from 0.25 mg/ml to 0.50 mg/ml.[83,106]

Corynebacterium diphtheriae, is a non-motile, non-encapsulated, Gram-positive bacterium and the leading cause of diphtheria infection. Diphtheria was previously considered a serious childhood disease, however, due to extensive vaccination programs, infections as a result of this pathogen are now limited with the majority of outbreaks identified within underdeveloped countries.[107] As of 2013, 4'680 diphtheria cases were reported to WHO, with an estimated 2'500 associated deaths.[108] Respiratory diphtheria presents as a grayish-white, fibrinous and firmly adherent pseudomembrane over the tonsils, the pharynx, or the larynx. Other clinical manifestations include hoarseness and stridor. Extension of the characteristic adherent pseudomembrane correlates with disease severity, while resultant suffocation is a common cause of death in this infection.[107] Despite the global occurrences of this pathogen and deaths associated, no studies have been conducted on the antimicrobial potential of essential oils against this micro-organism. The limits in scientific research may be due to the complexity of growth requirements for this pathogen for antimicrobial assay.

The vast majority of studies identified made use of antimicrobial susceptibility testing methods such as disk diffusion, which as previously stated, has been shown to be inconsistent and an unreliable measure of antimicrobial effect of essential oils. The results of the MIC antimicrobial studies undertaken and reviewed indicate potential for use of *Lavandula angustifolia*, *Mentha piperita*, *Thymus vulgaris*, *Zingiber officinale* and *Citrus limon* in the treatment of respiratory infections. Despite these findings, more than 50 essential oils are indicated for use in layman literature for the management and treatment of respiratory tract infections. To corroborate or refute these claims, it is suggested that further research be conducted on a greater number of essential oils, as indicated for respiratory use, for antimicrobial potential against respiratory specific pathogens.

The Antifungal Activity of Essential Oils Used for Respiratory Infections

Antifungal Mechanism of Action of Essential Oils

The mechanism of action of essential oils against fungi is very similar to that of bacterial cells with disruption of the cell architecture leading to a cascade of events responsible for cell death.[109] Essential oils have demonstrated potent antifungal effects due to the lipophilic nature of these products. Essential oils comprising primarily of chemical constituents of low molecular weight and increased lipophilicity have an increased antifungal effect.[109] Essential oils have the ability to disrupt the cell membrane of a fungal cell by binding to ergosterols, the highly lipophilic building blocks of the cell, responsible for maintaining cell fluidity and membrane integrity, while also preventing the production of beta-glucans, responsible for maintaining the fungal cell wall. Within the cell, essential oils have demonstrated the ability to cause dysfunction to the mitochondria leading to inhibition of proton pumps and reduction in ATP production, leading to cell death.[110] In addition, essential oils have demonstrated the ability to affect the organelle of the cell through the same mechanisms responsible for damage to the cell membrane.[109]

Within the fungi genus, 300 000 varying species exist, however, less than 1.0% of these organisms are parasitic and thus pathogenic to a human host.[111] Pathogenesis of fungi within human hosts occurs through truly pathogenic fungi and opportunistic fungi within immunocompromised patients. Respiratory-related infections caused by fungi and yeasts only occur within immunocompromised hosts and include pathogens such as *Aspergillus niger*, *Candida albicans* and *Cryptococcus neoformans*.

Aspergillus species, including *A. niger*, are widely identifiable molds sourced within the environment (including soil and air) and are responsible for pulmonary aspergillosis in severely immunocompromised hosts. Pulmonary aspergillosis is a critical condition caused by the inhalation of *Aspergillus* spores into the lower respiratory tract.[112] Treatment of pulmonary aspergillosis includes the intravenous use of the antifungal agent, Amphotericin B, however, mortality associated to this condition ranges from 50–90.0% in infected patients.[113] Essential oils investi-

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gated against this fungal pathogen proved to elicit noteworthy antimicrobial effect, with all essential oils reported having demonstrated an MIC value of less than 1.00 mg/ml (*Table 6*).[114–118]

The essential oil most effective was *Daucus carota* (carrot seed) with MIC values of between 0.01 mg/ml and 0.02 mg/ml against both clinical and reference strains of this pathogen.[115] This essential oil is indicated by aromatherapists for the treatment of upper and lower respiratory tract infections such as influenza and bronchitis, as well as in the management of coughs associated to these conditions.[41] Scientific studies on *D. carota* essential oil have demonstrated noteworthy antimicrobial effects against a number of other, non-respiratory related micro-organisms,[6,98] with greater antifungal activity noted then antibacterial activity. The antifungal effects are attributed to the virulent component of fungi (germ tube formation), being inhibited by *D. carota* essential oil.[119]

Candida albicans is a natural commensal of the upper respiratory tract within the human body and is rarely systemically pathogenic with Candida pneumonia considered a rare infection of the lung.[120] Candida pneumonia is only identified in patients on long-term antibiotic use, ventilation, or in severely immunocompromised and metastatic patients. Onset of infection often is because of Candida pathogenesis from a distant site such as the skin or gastrointestinal tract.[121,122] Treatment of *Candida* pneumonia includes the use of Amphotericin B intravenously; however, *Candida* pneumonia still maintains a high degree of mortality in all isolated patients.[123]

Cryptococcus neoformans is a fungal pathogen transmitted to human hosts by means of inhalation of *Cryptococcal* propagules. *Cryptococcus neoformans* typically affects immunocompromised hosts and is considered a life-threatening fungal infection with a high degree of mortality. *Cryptococcus neoformans* is the only known eukaryote with a polysaccharide capsule enveloping it. This capsule aids in evading host immunity and is significant in the virulence of this pathogen.[124,125] Essential oils tested against respiratory fungal pathogens have demonstrated noteworthy antimicrobial potential with 65.5% of the 13 essential oils investigated having an MIC value of 1.00 mg/ml or less.[67,68,70,71,74,115,117] As seen in *Table 6*, *Daucus carota* essential oil, once again, was determined as the oil with the greatest antimicrobial effect of the essential oils investigated with an MIC value of 0.01–0.02 mg/ml against *Candida albicans* and 0.0003–0.0006 mg/ml against *Cryptococcus neoformans*.[115] Similar findings were determined by Hammer et al.[98] and de Rapper et al.[6] Other essential oils with similar antifungal effects include *Cinnamomum zeylanicum*, *Cymbopogon citratus* (lemongrass), *Citrus limon*, *Lavandula angustifolia* and *Mentha piperita*.

The Antiviral Activity of Essential Oils Used for Respiratory Infections

Antiviral Mechanism of Action of Essential Oils

The antiviral mechanism of action of essential oils is predominantly understudied with very little known of the potential mechanisms of action.[53] Previous studies have reported the inhibition of virion envelopment, designed for viral entry into host cells, as well as the inhibition of virus replication by hindering cellular DNA polymerase and alteration in phenylpropanoid pathways.[126] Viruses present a complex challenge for essential oils, as some viral pathogens are encapsulated while others are not. The encapsulated viruses are often more resistant to the lipophilic nature of essential oils and as such present a unique challenge in prevention of viral cells.[127] Viruses are parasitic infections within susceptible host cells that cannot replicate themselves. Viruses act by initiating gene expression processes within host cells to create more of the virus.[128] The viruses most responsible for respiratory infection include rhinovirus, parainfluenza virus, coronavirus, adenovirus, respiratory syncytial virus, coxsackie virus, human metapneumovirus, and influenza virus, summarized in *Tables 1* and *2*.

The antiviral potential of essential oils has not been well-established within the scientific literature. Only one study could be identified on the effect of essential oils against the respiratory virus, coronavirus and two others against the human influenza virus.[129–131] The coronavirus is a virus commonly isolated within human populations with 15–30.0% of respiratory cases identified, annually.[132] Populations most at risk for infection include neonates, the elderly and immunocompromised individuals. Respiratory infections caused by this virus are self-limiting with no known antiviral or preventative vaccination available.[132] According to Loizzo et al.,[129] an IC_{50} value is determined when evaluating the antiviral activity of a sample. This value indicates the effective concentration of a sample responsible for inhibiting 50.0% of virus growth. Further to this, the selective index (SI) may also be provided as a means of antiviral potential measurement. An essential oil with a SI value greater than four is considered suitable as an antiviral

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Table 6. Essential oil studies showing efficacy against fungal pathogens associated with respiratory tract infections.

Essential oil Scientific name *Aspergillus* species	Common name	Pathogen strain	Result (MIC)	Main chemical constituent	Reference
Cinnamomum zeylanicum	Cinnamon	clinical isolate	0.25 µl/ml	Cinnamaldehyde (62.79%)	[114]
Daucus carota	Carrot seed	ATCC 16404	0.01 mg/ml	α-Pinene (37.9%)	[115]
		ATCC 16404	0.02 mg/ml	Geranyl acetate (65.0%)	[115]
		ATCC 16404	0.01 mg/ml	Carotol (25.1%)	[115]
		ATCC 16404	0.01 mg/ml	β-Bisabolene (51.0%)	[115]
Laurus nobilis	Bay	clinical isolate	40.0 µl/ml	1,8-Cineole (41.86%)	[114]
Candida albicans					
Coriandrum sativum	Coriander	clinical isolate	163.0 mg/ml	Decanal (14.3%)	[65]
		CBS 562	0.50 mg/ml	1-Decanol (24.2%)	[116]
Daucus carota	Carrot seed	ATCC 10231	0.01 mg/ml	α-Pinene (37.9%)	[115]
		ATCC 10231	> 0.02 mg/ml	Geranyl acetate (65.0%)	[115]
		ATCC 10231	> 0.02 mg/ml	Carotol (25.1%)	[115]
		ATCC 10231	0.01 mg/ml	β-Bisabolene (51.0%)	[115]
Eucalyptus globulus	Eucalyptus	ATCC 90028	10.0 mg/ml	1,8-Cineole (81.9%)	[67]
Juniperus communis	Juniper berry	ATCC 90028	20.0 mg/ml	α-Pinene (398.%)	[67]
Lavandula angustifolia	Lavender	ATCC 10231	0.25 mg/ml	Linalool (40.3%)	[68]
		ATCC 10231	0.75 mg/ml	Linalool (57.1%)	[69]
Lavandula intermedia	Lavandin	ATCC 10231	0.75 mg/ml	Linalool (54.0%)	[69]
Melaleuca alternifolia	Tea tree	ATCC 10231	6.0 mg/ml	n.d.[a]	[70]
Melaleuca leucadendra	Cajuput	ATCC 90028	2.50 mg/ml	1,8-Cineole (67.6%)	[67]
Mentha piperita	Peppermint	ATCC 10231	0.11 mg/ml	Menthol (47.5%)	[68]
		ATCC 10231	6.0 mg/ml	n.d.	[70]
		ATCC 90028	0.30 mg/ml	Menthol (47.3%)	[67]
		clinical isolate	0.625 mg/ml	Menthone (27.9%)	[71]
		clinical isolate	0.625 mg/ml	Menthol (42.3%)	[71]
		clinical isolate	0.312 mg/ml	Menthol (39.9%)	[71]
		clinical isolate	0.625 mg/ml	Menthol (34.6%)	[71]
Myrtus communis	Myrtle	clinical isolate	8.00 µl/ml	1,8-Cineole (36.10%)	[117]
Rosmarinus officinalis	Rosemary	ATCC 10231	6.0 mg/ml	n.d.	[70]
Thymus vulgaris	Thyme	ATCC 10231	3.3 mg/ml	n.d.	[70]
Cryptococcus neoformans					
Citrus limon	Lemon	ATCC 14116	0.83 mg/ml	n.d.	[64]
Daucus carota	Carrot seed	CECT 1078	0.0003 mg/ml	α-Pinene (37.9%)	[115]
		CECT 1078	0.0006 mg/ml	Geranyl acetate (65.0%)	[115]
		CECT 1078	0.0003 mg/ml	Carotol (25.1%)	[115]
		CECT 1078	0.0006 mg/ml	β-Bisabolene (51.0%)	[115]
Lavandula angustifolia	Lavender	Clinical strain	0.75 mg/ml	Linalool (57.1%)	[69]
Lavandula intermedia	Lavandin	Clinical strain	0.75 mg/ml	Linalool (54.0%)	[69]

[a] Not determined.

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agent,[129,133] while an IC_{50} value of less than 0.001 mg/ml is considered as noteworthy.[129,133] With this in mind, none of the essential oils investigated (*Cupressus sempervirens* (cypress), *Juniperus oxycedrus*, *Laurus nobilis* (bay laurel) and *Salvia officinalis* (sage)) against the coronavirus isolate indicated noteworthy activity, however, *L. nobilis* essential oil did produce an SI value of 4.20 indicating potential activity.[129] *Laurus nobilis* is indicated within the layman aroma-therapeutic literature for the treatment of lower respiratory tract infections such as bronchitis, an acute condition often associated to viral infections.[41] *Laurus nobilis* essential oil has been shown to have positive antiviral effects against viruses associated with plants and animals and reduces the viral load and virus replication.[134,135]

The incidence of human influenza virus amounts to 8.0% of respiratory infections, annually.[136] Influenza symptoms include sudden onset of fever, muscle pain, headaches and exhaustion, often resolving within 1–2 weeks.[137] Populations most at risk for infection include infants, older adults, pregnant women, and the immunocompromised. Respiratory infections caused by this virus are self-limiting, however, seasonal preventative vaccinations are available and updated annually.[137]

According to a study conducted by Garozzo et al.[131] the essential oil of *Melaleuca alternifolia* (tea tree) has an inhibitory effect on influenza virus replication at doses below the cytotoxic dose. This study determined that viral replication was significantly inhibited if tea tree essential oil was added in the early stages of infection (within two hours of infection), indicating that tea tree essential oil inhibits viral replication of influenza virus at the early phase of replication.[131] In another study against the human influenza virus, essential oils were combined for an antiviral effect.[130] In this study, the essential oils of *Coridothymus capitatus* L. (Spanish oregano), *Origanum dictamnus* L. (dittany) and *Salvia fruticosa* Mill. (Greek sage) were added to extra-virgin olive oil and tested against human influenza virus strains. The study revealed that the essential oil combination displayed antiviral activity against influenza A/H1N1 virus strains, influenza B and human rhinovirus 14 (HRV14), while no viral inhibition was found against influenza A/H3N2, respiratory syncytial virus (RSV) and adenovirus 5.

No other data is available on the effects of essential oils alone or in combination against human viral species relating to respiratory tract infections.

Essential Oil Chemistry

Essential oil chemistry has been well-documented within the literature with researchers identifying various essential oil chemotypes related to biological activity. Essential oil chemistry correlates strongly to essential oil quality and is governed by several institutions such as the European Pharmacopoeia and the International Standard Organization (ISO).[138] From the literature, it has been determined that essential oils can comprise of up to more than sixty different compounds, however, essential oils predominate in only a few major constituents.[138] These major chemical compounds can constitute up to 85.0% of the essential oil with the other individual compounds identified comprising in trace amounts.[56] The antimicrobial activity is very often as a result of the synergistic effects of the total interaction of chemical compounds constituting the essential oil. Numerous studies have identified that perceived biological activity of an essential oil may be more closely related to the activity of the major chemical constituent.[138–141] Essential oils containing aldehydes or phenols such as citral, carvacrol, eugenol or thymol as major compound showed the highest degree of antibacterial activity, while essential oils comprising of ketone or esters such as β-myrcene, α-thujone or geranyl acetate have much weaker activity.[142,143] *Table 7* demonstrates the major chemical compounds per essential oil used for respiratory infections. Of the essential oils investigated, 96.0% of the major chemical constituents identified are of terpenoid origin, of which 78.0% are monoterpenes.

The antimicrobial activities of some essential oil chemical compounds associated with essential oils used to treat respiratory infections are highlighted in *Table 8*.

According to Rios and Recio[37] noteworthy antimicrobial activity is considered for compounds exhibiting an MIC values less than or equivalent to 0.01 mg/ml, while it is further stated by Gibbons[186] that compounds demonstrating an MIC value of between 0.06 and 0.10 mg/ml are of clinical relevance. On critical examination, the chemical compounds as elaborated on in *Table 8*, demonstrated very little noteworthy antimicrobial effects, according to standards defined by Gibbons.[186] These compounds include, carvacrol against *S. aureus* (MIC = 0.002 mg/ml) and *P. aeruginosa* (MIC = 0.005 mg/ml), menthol against *S. aureus* (MIC = 0.01 mg/ml) and thymol against *M. tuberculosis* (MIC = 0.008 mg/ml), *S. aureus* (0.003 mg/ml) and *P. aeruginosa* (MIC = 0.01 mg/ml). *Origanum vulgare* (comprising of carvacrol), *Mentha piperita* (comprising of menthol) and *T. vulgaris* (comprising of thymol-as main constituents). These

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Table 7. The major chemical composition of the essential oils commonly used in respiratory tract infections.

Major constituent/s	Constituent class	Essential oil Scientific name	Common name	Reference
trans-Anethole	Monoterpene	*Foeniculum dulce*	Fennel	[6]
Anethole	Monoterpene	*Foeniculum vulgare*	Fennel	[144]
Camphor	Monoterpene	*Cinnamomum camphora*	Camphor	[145]
		Rosmarinus officinalis	Rosemary	[6]
		Salvia officinalis	Sage	[145]
Carotol	Sesquiterpene	*Daucus carota*	Carrot seed	[6,115,146]
Carvacrol	Monoterpene	*Origanum vulgare*	Origanum	[145]
Carvone	Monoterpene	*Carum carvi*	Caraway	[6,147]
β-Caryophyllene	Sesquiterpene	*Piper nigrum*	Black pepper	[6,148]
α-Cedrene	Sesquiterpene	*Cedrus atlantica*	Cedarwood	[6]
		Juniperus sp.	Juniper	[149]
1,8-Cineole	Monoterpene	*Elettaria cardamomum*	Cardamom	[145]
		Eucalyptus globulus	Eucalyptus	[6,150]
		Lavandula latifolia	Lavender spike	[145]
		Melaleuca viridiflora	Niaouli	[6,151]
		Myrtus communis	Myrtle	[6,152]
		Origanum majorana	Marjoram	[6,153]
		Rosmarinus officinalis	Rosemary	[145]
Cinnamyl alcohol	Monoterpene	*Styrax benzoin*	Benzoin	[6,154]
Citronellol	Monoterpene	*Rosa* × *damascena*	Rose	[145]
Diallyl trisulfide	Other	*Allium sativum*	Garlic	[145]
Eugenol	Monoterpene	*Cinnamomum zeylanicum*	Cinnamon	[6,155]
		Laurus nobilis	Bay	[6,156]
		Pimenta dioica	Pimento	[145,157]
		Syzygium aromaticum	Clove	[6]
Furanoeudesma-1,3-diene	Sesquiterpene	*Commiphora myrrha*	Myrrh	[6,158]
Geranial	Monoterpene	*Cymbopogon citratus*	Lemongrass	[6,159]
Isopinocamphone	Monoterpene	*Hyssopus officinalis*	Hyssop	[6,160]
Limonene	Monoterpene	*Canarium luzonicum*	Elemi	[6,161]
		Citrus aurantifolia	Lime	[145]
		Citrus bergamia	Bergamot	[145]
		Citrus limon	Lemon	[6,162]
		Citrus sinensis	Orange	[6,163]
Linalool	Monoterpene	*Coriandrum sativum*	Coriander	[145]
		Lavandula intermedia	Lavandin	[145]
		Ocimum basilicum	Basil	[6,164]
Linalyl acetate	Monoterpene	*Lavandula angustifolia*	Lavender	[6,165]
		Salvia sclarea	Clary Sage	[6,166]
Menthol	Monoterpene	*Mentha piperita*	Peppermint	[6,167]
Terpinen-4-ol	Monoterpene	*Melaleuca alternifolia*	Tea tree	[6,168]
α-Pinene	Monoterpene	*Cupressus sempervirens*	Cypress	[6,169]
		Helichrysum italicum	Immortelle	[145]
		Pinus sylvestris	Pine	[145]
β-Pinene	Monoterpene	*Abies balsamea*	Fir	[6,170]
Thymol	Monoterpene	*Thymus vulgaris*	Thyme	[6,171]
Zingiberene	Sesquiterpene	*Zingiber officinale*	Ginger	[145]

[a] Not determined.

essential oils have demonstrated the greatest degree of antimicrobial effect against pathogens of the respiratory tract, thus supplementing claims that the perceived biological activity of an essential oil may be more closely related to the activity of the major chemical constituent. It is prudent to consider that the interaction of chemical constituents within an essential oil create a synergistic environment that produces an antimicrobial effect by

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Table 8. The antimicrobial activity of major chemical composition within essential oils used in respiratory tract infections.

Major constituent/s	Antimicrobial activity (MIC)		Reference
trans-Anethole	Antibacterial		
	M. tuberculosis (H37Rv)	20.83 μg/ml	[172]
	K. pneumoniae (RSKK 574)	8 μg/ml	[89]
Camphor	Antibacterial		
	M. tuberculosis (H37Rv)	41.66 μg/ml	[172]
	S. aureus (ATCC 25923)	0.06 mg/ml	
	P. aeruginosa (ATCC 27853)	0.07 mg/ml	
	S. pneumoniae (clinical isolate)	8.00 mg/ml	[173]
Camphor	Antiviral		
	Influenza A (H1N1a)	EC_{50}= 4.1–1644.00 μM	[174]
	Influenza A (H1N1a)	EC_{50}= 4.8–1447.00 μM	[175]
Carotol	Not known		
Carvacrol	Antibacterial		
	M. tuberculosis (H37Rv)	2.02 μg/ml	[172]
	S. aureus (CCMI 335)	0.10 mg/ml	[74]
	S. aureus (ATCC 25923)	0.01 mg/ml	
	S. aureus (ATCC 25923)	0.51 mg/ml	
	S. aureus (ATCC 29213)	0.51 mg/ml	
	S. aureus (ATCC 33591)	0.97 mg/ml	
	S. aureus (ATCC 33592)	0.68 mg/ml	
	S. aureus (ATCC 43300)	0.48 mg/ml	
	S. aureus (clinical isolate)	0.57 – 1.02 mg/ml	
	P. aeruginosa (CCMI 331)	>0.20 mg/ml	[74]
	P. aeruginosa (ATCC 27853)	0.01 mg/ml	
	K. pneumoniae (RSKK 574)	8 μg/ml	[89]
Carvacrol	Antifungal		
	C. albicans (CCMI 209)	0.05 mg/ml	[74]
Carvone	Antibacterial		
	M. tuberculosis (H37Rv)	20.83 μg/ml	[172]
	K. pneumoniae (RSKK 574)	8 μg/ml	[89]
β-Caryophyllene	Antibacterial		
	M. tuberculosis (H37Rv)	100 μg/ml	[172]
α-Cedrene	Antifungal		
	C. albicans (clinical isolate)	<0.05 mg/ml	[176]
1,8-Cineole	Antibacterial		
	S. aureus (ATCC 25923)	0.05 mg/ml	[177]
	P. aeruginosa (ATCC 27853)	0.07 mg/ml	
	S. pneumoniae (clinical isolate)	2.50 mg/ml	[173]
1,8-Cineole	Antifungal		
	C. albicans (ATCC 10231)	1.00 mg/ml	[178]
	C. albicans (clinical isolate)	4.00% (v/v)	[179]
Cinnamyl alcohol	Antibacterial		
	S. aureus (clinical isolate)	0.25 mg/ml	[180]
	P. aeruginosa (clinical isolate)	1.00 mg/ml	[180]
Citronellol	Antibacterial		
	K. pneumoniae (RSKK 574)	8 μg/ml	[89]
Diallyl trisulfide	Antibacterial		
	S. pyogenes (clinical isolate)	0.02 mg/ml	[181]
Eugenol	Antibacterial		

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Table 8. (cont.)

Major constituent/s	Antimicrobial activity (MIC)		Reference
	M. tuberculosis (H37Rv)	25 μg/ml	[173]
	K. pneumoniae (RSKK 574)	8 μg/ml	[89]
Furanoeudesma-1.3-diene	Not known		
Geranial	Antibacterial		
	M. tuberculosis (H37Rv)	12.5 μg/ml	[172]
Isopinocamphone	Not known		
Limonene	Antibacterial		
	M. tuberculosis (H37Rv)	25 μg/ml	[172]
	K. pneumoniae (RSKK 574)	8 μg/ml	[89]
	S. aureus (ATCC 25923)	0.08 mg/ml	
	P. aeruginosa (ATCC 27853)	0.10 mg/ml	
	S. pneumoniae (clinical isolate)	> 16.00 mg/ml	[173]
Limonene	Antifungal		
	C. albicans (clinical isolate)	> 8.00 % (v/v)	[179]
Linalool	Antibacterial		
	M. tuberculosis (H37Rv)	33.33 μg/ml	[172]
	K. pneumoniae (RSKK 574)	8 μg/ml	[89]
	K. pneumoniae (ATCC 10031)	1.50 mg/ml	
	S. pneumoniae (clinical isolate)	6.00 mg/ml	[173]
	S. aureus (NTCC 6571)	0.25 % (v/v)	[178]
	S. aureus (ATCC 25923)	1.50 mg/ml	
	S. pyogenes (ATCC 12204)	0.75 mg/ml	
Linalool	Antifungal		
	C. albicans (ATCC 10231)	0.25 % (v/v)	[178]
	C. albicans (ATCC 10231)	0.50 mg/ml	[179]
	C. albicans (ATCC 90028)	0.025 % (v/v)	[182]
	C. albicans (clinical isolate)	0.10 mg/ml	[183]
	C. neoformans (clinical isolate)	0.25 mg/ml	
Linalyl acetate	Antibacterial		
	K. pneumoniae (ATCC 10031)	25.00 mg/ml	
	P. aeruginosa (ATCC 27853)	0.10 mg/ml	
	S. aureus (ATCC 25923)	1.25 mg/ml	
	S. aureus (ATCC 25923)	2.50 mg/ml	
	S. aureus (ATCC 6538P)	0.08 mg/ml	[184]
	S. pyogenes (ATCC 12204)	2.50 mg/ml	
Linalyl acetate	Antifungal		
	C. albicans (ATCC 10231)	15.00 mg/ml	
	C. neoformans (clinical isolate)	2.50 mg/ml	
Menthol	Antibacterial		
	M. tuberculosis (H37Rv)	41.66 μg/ml	[172]
	S. pneumoniae (clinical isolate)	1.50 mg/ml	[173]
	S. aureus (ATCC 6538)	0.625 mg/ml	[71]
	S. aureus (ATCC 25923)	0.01 mg/ml	
	S. aureus (ATCC 6538P)	0.62 mg/ml	[184]
	P. aeruginosa (ATCC 27853)	0.03 – 2.50 mg/ml	[71]
	K. pneumoniae (RSKK 574)	8 μg/ml	[89]
	K. pneumoniae (clinical isolate)	2.5 mg/ml	[71]
Menthol	Antifungal		
	C. albicans (clinical sample)	0.625 mg/ml	[71]
Terpinen-4-ol	Antibacterial		

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Table 8. (cont.)

Major constituent/s	Antimicrobial activity (MIC)		Reference
	S. aureus (NTCC 6571)	0.25% (v/v)	[178]
Terpinen-4-ol	Antifungal		
	C. albicans (clinical isolate)	0.25% (v/v)	[179]
α-Pinene	Antibacterial		[185]
	K. pneumoniae (RSKK 574)	8 μg/ml	[89]
	S. aureus (ATCC 25923)	0.06 mg/ml	
	P. aeruginosa (ATCC 27853)	0.10 mg/ml	[183]
α-Pinene	Antifungal		
	C. albicans (clinical isolate)	0.30 mg/ml	[179, 183]
β-Pinene	Antibacterial		
	M. tuberculosis (H37Rv)	10.41 μg/ml	[172]
	K. pneumoniae (RSKK 574)	8 μg/ml	[89]
	K. pneumoniae (clinical isolate)	0.10 mg/ml	
β-Pinene	Antifungal		
	C. albicans (clinical isolate)	0.01% (v/v)	[179]
Thymol	Antibacterial		
	M. tuberculosis (H37Rv)	0.78 μg/ml	[172]
	S. aureus (CCMI 335)	0.10 mg/ml	[74]
	S. aureus (ATCC 6538P)	1.25 mg/ml	[184]
	S. aureus (ATCC 25923)	0.45 mg/ml	
	S. aureus (ATCC 29213)	0.51 mg/ml	
	S. aureus (ATCC 33591)	1.02 mg/ml	
	S. aureus (ATCC 33592)	0.68 mg/ml	
	S. aureus (ATCC 43300)	0.48 mg/ml	
	S. aureus (clinical isolate)	0.51–1.02 mg/ml	
	P. aeruginosa (CCMI 331)	0.20 mg/ml	[74]
	P. aeruginosa (ATCC 27853)	0.01 mg/ml	
	K. pneumoniae (RSKK 574)	8 μg/ml	[89]
Thymol	Antifungal		
	C. albicans (CCMI 209)	0.10 mg/ml	[74]
Zingiberene	Not known		

the essential oil. Such findings have been determined by previously published investigations.[187,188] Previous studies have reported that the inhibitory action of *Rosmarinus officinalis* (rosemary) essential oil against larvae insect is due to combined effects of various chemical constituents as further investigation indicated that no single chemical constituent showed any biological activity.[140,189]

Additional research, making use of studies such as chemometrics, will allow for researchers to determine the link between chemistry and antimicrobial activity. Chemometrics is the application of mathematical and statistical techniques used to retrieve significant information from complex datasets.[190] Chemometrics is an invaluable tool for identifying patterns and determining structure–activity relationships within complex biological systems and offers an unbiased approach to review of these interactions. Studies using chemometrics to determine structure-activity relationships of essential oils for antimicrobial purposes have demonstrated important findings of essential oil chemistry and its correlation to antimicrobial activity.[190,191] In a study conducted by Orchard et al.,[191] it was determined that the chemical compound eugenol was identified as the biomarker that contributes to the noteworthy antibacterial activity of essential oils such as *C. zeylanicum*, *L. nobilis* and *S. aromaticum* against dermatological pathogens. This link should be explored to determine the effect of essential oil chemistry on antimicrobial activity and the ability to use this data to predict synergy in essential oil combinations.

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Essential Oils Used in Combination for Respiratory Applications

Aromatherapy predominantly makes use of essential oils in combination. Essential oil blending, or the combining of essential oils, is the heart of aromatherapeutic practice, with the aim of creating synergistic interactions between oils.[192] Synergy is a concept that has been conceived since antiquity and actively sought within a vast array of scientific fields. Synergy can be defined as an effect seen by a combination of substances being greater than would have been expected from the individual contributions.[193] Synergy within the field of aromatherapy can be achieved if the essential oils are able to elicit antimicrobial effects at varying target sites, or by enhancing the bioavailability of one or more oils within the combination.[50]

Synergy is measured using a mathematical definition dependent on the technique used for quantification.[193] Multiple techniques are currently employed to measure synergy, dependent on the components analyzed. For the antimicrobial analysis, the calculation of interaction indices and creation of isobolograms are the accepted methods used to interpret multiple interactions. In the calculation of an interactive index known as the Fractional Inhibitory Index (IFIC), the results obtained from the MIC and MBC assays are used. The interpretation of results obtained by the calculation are; synergy (≤ 0.5), additive effects ($>0.5-1.0$), non-interactive or indifferent effects ($>1.0-\leq 4.0$), or antagonistic effects (>4.0).[51] The IFIC is a mathematical evaluation of the interaction of tested combinations in order to determine possible synergy.[51] The limitation to this method is the assumption that half the concentration will only offer half the effect.[50,194] In order to overcome this, interactions at varying concentrations can be determined and represented in isobolograms, which are graphical illustrations of combined interactions. Proposed combinations are evaluated independently and at varying concentrations to develop a dose response plot.[51] Points identified below the 0:50:0.50 line of the isobologram are considered synergistic.[51]

Essential Oils in Combination with Essential Oils

Examining the aroma-therapeutic literature, at least 445 possible combinations (made up of two oils), could be identified for the treatment of respiratory tract infections.[7,38,41–45] When investigating the scientific literature on the antimicrobial effect of these identified blends against respiratory specific pathogens, very few combinations were studied (*Table 9*).[6,195–202]

Although there has been a surge in interest and encouraged direction in the field of combinational essential oil research, data (*Figure 6*) demonstrates that research involving combinations has remained consistently lower (approximately half) that of studies on single oils for the last 30 years. This is vastly unbalanced considering that essential oil blending is the primary based application.

The data summarized in *Table 9* demonstrates that essential oils, when used in combination, can initiate a synergistic antimicrobial effect. Synergy was determined for 34.0% of the combinations reviewed. The combination of essential oils proving the most synergistic antimicrobial effect is the combination of *Lavandula angustifolia* and *Litsea cubeba* (May Chang) in which an IFIC value of 0.19 was obtained against the pathogen *Candida albicans*.[6] Combinations identifying as synergistic against a specific pathogen may not demonstrate the same effect against a different pathogen of the same or different class. This is highlighted in the combination of *Lavandula angustifolia* and *Litsea cubeba* essential oils in where the combination demonstrates synergy against the pathogen *C. albicans* but is additive against *Pseudomonas aeruginosa* and non-interactive against *Staphylococcus aureus*.[6] These antimicrobial effects are predominant (83.1% of the combinations studied), with combinations demonstrating synergy against one pathogen and differing antimicrobial effects against microorganisms of differing classes. This demonstrates that broad-spectrum synergy cannot be assumed, and that combinations should be tested for specific infectious conditions against specific condition-related pathogens. This is particularly important in the case of aromatherapy when using essential oils to treat and manage respiratory infections. Multiple causative agents are responsible for these conditions, as highlighted in *Tables 1* and *2*, and as such, aromatherapists should not assume efficacy of essential oils broadly in the treatment of respiratory related infections due to the complexity of these conditions.

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Table 9. Essential oil studies showing efficacy of essential oils combinations against pathogens of the respiratory tract.

Essential oil	Pathogen strain	FIC	Result[b]	Reference
Cinnamomum zeylanicum (cinnamon) + *Syzygium aromaticum* (clove)	*S. aureus* (ATCC 29213)	1.80	I	[195]
Cinnamomum zeylanicum (cinnamon) + *Thymus vulgaris* (thyme)	*S. aureus* (ATCC 25923)	0.26	S	[196]
Cymbopogon citratus (lemongrass) + *Cymbopogon giganteus* (lemongrass)	*S. aureus* (ATCC 9144)	0.4	S	[197]
Mentha piperita (peppermint) + *Ocimum basilicum* (basil)	*S. aureus* (ATCC 9144)	0.36	S	[198]
Ocimum basilicum (basil) + *Citrus bergamia* (bergamot)	*S. aureus* (ATCC 6538)	0.38	S	[199]
Origanum vulgare (origanum) + *Citrus bergamia* (bergamot)	*S. aureus* (ATCC 6538)	0.38	S	[199]
Origanum vulgare (origanum) + *Ocimum basilicum* (basil)	*S. aureus* (ATCC 6538)	0.38	S	[199]
Origanum vulgare (origanum) + *Ocimum basilicum* (basil)	*P. aeruginosa* (ATCC 27853)	1.00	A	[200]
Origanum vulgare (origanum) + *Origanum majorana* (majoram)	*P. aeruginosa* (ATCC 27853)	1.75	I	[200]
Origanum vulgare (origanum) + *Rosmarinus officinalis* (rosemary)	*P. aeruginosa* (ATCC 27853)	1.50	I	[200]
Origanum vulgare (origanum) + *Salvia triloba* (sage)	*P. aeruginosa* (ATCC 27853)	1.50	I	[200]
Origanum vulgare (origanum) + *Thymus vulgaris* (thyme)	*P. aeruginosa* (ATCC 27853)	0.88	A	[200]
Origanum vulgare (origanum) + *Rosmarinus officinalis* (rosemary)	*S. aureus* (clinical isolate)	0.50	S	[201]
Syzygium aromaticum (clove) + *Rosmarinus officinalis* (rosemary)	*S. aureus* (ATCC 6538)	n.m.[b]	A	[202]
Syzygium aromaticum (clove) + *Rosmarinus officinalis* (rosemary)	*P. aeruginosa* (ATCC 27853)	n.m.	A	[202]
Syzygium aromaticum (clove) + *Rosmarinus officinalis* (rosemary)	*C. albicans* (ATCC 10231)	n.m.	S	[202]
Lavandula angustifolia (lavender) + *Canarium luzonicum* (elemi)	*C. albicans* (ATCC 10231)	0.25	S	[6]
Lavandula angustifolia (lavender) + *Canarium luzonicum* (elemi)	*S. aureus* (ATCC 6538)	3.33	I	[6]
Lavandula angustifolia (lavender) + *Canarium luzonicum* (elemi)	*P. aeruginosa* (ATCC 27858)	0.53	A	[6]
Lavandula angustifolia (lavender) + *Carum carvi* (caraway)	*C. albicans* (ATCC 10231)	0.42	S	[6]
Lavandula angustifolia (lavender) + *Carum carvi* (caraway)	*S. aureus* (ATCC 6538)	1.00	A	[6]
Lavandula angustifolia (lavender) + *Carum carvi* (caraway)	*P. aeruginosa* (ATCC 27858)	0.56	A	[6]
Lavandula angustifolia (lavender) + *Cinnamomum zeylanicum* (cinnamon)	*C. albicans* (ATCC 10231)	0.40	S	[6]
Lavandula angustifolia (lavender) + *Cinnamomum zeylanicum* (cinnamon)	*S. aureus* (ATCC 6538)	0.50	S	[6]
Lavandula angustifolia (lavender) + *Cinnamomum zeylanicum* (cinnamon)	*P. aeruginosa* (ATCC 27858)	0.53	A	[6]
Lavandula angustifolia (lavender) + *Citrus aurantium* (lime)	*C. albicans* (ATCC 10231)	0.42	S	[6]
Lavandula angustifolia (lavender) + *Citrus aurantium* (lime)	*S. aureus* (ATCC 6538)	1.13	I	[6]
Lavandula angustifolia (lavender) + *Citrus aurantium* (lime)	*P. aeruginosa* (ATCC 27858)	0.51	A	[6]
Lavandula angustifolia (lavender) + *Citrus medica limonum* (lemon)	*C. albicans* (ATCC 10231)	0.42	S	[6]
Lavandula angustifolia (lavender) + *Citrus medica limonum* (lemon)	*S. aureus* (ATCC 6538)	2.50	I	[6]
Lavandula angustifolia (lavender) + *Citrus medica limonum* (lemon)	*P. aeruginosa* (ATCC 27858)	0.52	A	[6]
Lavandula angustifolia (lavender) + *Citrus sinensis* (orange, Florida)	*C. albicans* (ATCC 10231)	0.42	S	[6]
Lavandula angustifolia (lavender) + *Citrus sinensis* (orange, Florida)	*S. aureus* (ATCC 6538)	0.38	S	[6]
Lavandula angustifolia (lavender) + *Citrus sinensis* (orange, Florida)	*P. aeruginosa* (ATCC 27858)	0.51	A	[6]
Lavandula angustifolia (lavender) + *Commiphora myrrha* (myrrh)	*C. albicans* (ATCC 10231)	0.29	S	[6]
Lavandula angustifolia (lavender) + *Commiphora myrrha* (myrrh)	*S. aureus* (ATCC 6538)	1.00	A	[6]
Lavandula angustifolia (lavender) + *Commiphora myrrha* (myrrh)	*P. aeruginosa* (ATCC 27858)	1.03	I	[6]
Lavandula angustifolia (lavender) + *Cupressus sempervirens* (cypress)	*C. albicans* (ATCC 10231)	0.15	S	[6]
Lavandula angustifolia (lavender) + *Cupressus sempervirens* (cypress)	*S. aureus* (ATCC 6538)	0.58	A	[6]
Lavandula angustifolia (lavender) + *Cupressus sempervirens* (cypress)	*P. aeruginosa* (ATCC 27858)	0.53	A	[6]
Lavandula angustifolia (lavender) + *Cymbopogon citratus* (lemongrass)	*C. albicans* (ATCC 10231)	6.67	An	[6]
Lavandula angustifolia (lavender) + *Cymbopogon citratus* (lemongrass)	*S. aureus* (ATCC 6538)	0.55	A	[6]
Lavandula angustifolia (lavender) + *Cymbopogon citratus* (lemongrass)	*P. aeruginosa* (ATCC 27858)	0.52	A	[6]

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Table 9. (cont.)

Essential oil	Pathogen strain	FIC	Result[b]	Reference
Lavandula angustifolia (lavender) + *Daucus carota* (carrot seed)	*C. albicans* (ATCC 10231)	0.50	S	[6]
Lavandula angustifolia (lavender) + *Daucus carota* (carrot seed)	*S. aureus* (ATCC 6538)	0.50	S	[6]
Lavandula angustifolia (lavender) + *Daucus carota* (carrot seed)	*P. aeruginosa* (ATCC 27858)	0.56	A	[6]
Lavandula angustifolia (lavender) + *Eucalyptus globulus* (eucalyptus)	*C. albicans* (ATCC 10231)	0.38	S	[6]
Lavandula angustifolia (lavender) + *Eucalyptus globulus* (eucalyptus)	*S. aureus* (ATCC 6538)	1.50	I	[6]
Lavandula angustifolia (lavender) + *Eucalyptus globulus* (eucalyptus)	*P. aeruginosa* (ATCC 27858)	0.53	A	[6]
Lavandula angustifolia (lavender) + *Foeniculum dulce* (fennel)	*C. albicans* (ATCC 10231)	0.45	S	[6]
Lavandula angustifolia (lavender) + *Foeniculum dulce* (fennel)	*S. aureus* (ATCC 6538)	2.00	I	[6]
Lavandula angustifolia (lavender) + *Foeniculum dulce* (fennel)	*P. aeruginosa* (ATCC 27858)	0.52	A	[6]
Lavandula angustifolia (lavender) + *Hyssopus officinalis* (hyssop)	*C. albicans* (ATCC 10231)	0.33	S	[6]
Lavandula angustifolia (lavender) + *Hyssopus officinalis* (hyssop)	*S. aureus* (ATCC 6538)	1.67	I	[6]
Lavandula angustifolia (lavender) + *Hyssopus officinalis* (hyssop)	*P. aeruginosa* (ATCC 27858)	0.52	A	[6]
Lavandula angustifolia (lavender) + *Laurus nobilis* (bay)	*C. albicans* (ATCC 10231)	0.83	A	[6]
Lavandula angustifolia (lavender) + *Laurus nobilis* (bay)	*S. aureus* (ATCC 6538)	1.70	I	[6]
Lavandula angustifolia (lavender) + *Laurus nobilis* (bay)	*P. aeruginosa* (ATCC 27858)	0.60	A	[6]
Lavandula angustifolia (lavender) + *Litsea cubeba* (May Chang)	*C. albicans* (ATCC 10231)	0.19	S	[6]
Lavandula angustifolia (lavender) + *Litsea cubeba* (May Chang)	*S. aureus* (ATCC 6538)	1.17	I	[6]
Lavandula angustifolia (lavender) + *Litsea cubeba* (May Chang)	*P. aeruginosa* (ATCC 27858)	0.52	A	[6]
Lavandula angustifolia (lavender) + *Melaleuca alternifolia* (tea tree)	*C. albicans* (ATCC 10231)	0.50	S	[6]
Lavandula angustifolia (lavender) + *Melaleuca alternifolia* (tea tree)	*S. aureus* (ATCC 6538)	0.63	A	[6]
Lavandula angustifolia (lavender) + *Melaleuca alternifolia* (tea tree)	*P. aeruginosa* (ATCC 27858)	0.51	A	[6]
Lavandula angustifolia (lavender) + *Melaleuca viridiflora* (niaouli)	*C. albicans* (ATCC 10231)	0.90	A	[6]
Lavandula angustifolia (lavender) + *Melaleuca viridiflora* (niaouli)	*S. aureus* (ATCC 6538)	2.00	I	[6]
Lavandula angustifolia (lavender) + *Melaleuca viridiflora* (niaouli)	*P. aeruginosa* (ATCC 27858)	0.51	A	[6]
Lavandula angustifolia (lavender) + *Mentha piperita* (peppermint, China)	*C. albicans* (ATCC 10231)	0.63	A	[6]
Lavandula angustifolia (lavender) + *Mentha piperita* (peppermint, China)	*S. aureus* (ATCC 6538)	0.75	A	[6]
Lavandula angustifolia (lavender) + *Mentha piperita* (peppermint, China)	*P. aeruginosa* (ATCC 27858)	0.51	A	[6]
Lavandula angustifolia (lavender) + *Myrtus communis* (myrtle)	*C. albicans* (ATCC 10231)	0.50	S	[6]
Lavandula angustifolia (lavender) + *Myrtus communis* (myrtle)	*S. aureus* (ATCC 6538)	4.00	An	[6]
Lavandula angustifolia (lavender) + *Myrtus communis* (myrtle)	*P. aeruginosa* (ATCC 27858)	0.51	A	[6]
Lavandula angustifolia (lavender) + *Ocimum basilicum* (basil)	*C. albicans* (ATCC 10231)	0.67	A	[6]
Lavandula angustifolia (lavender) + *Ocimum basilicum* (basil)	*S. aureus* (ATCC 6538)	0.58	A	[6]
Lavandula angustifolia (lavender) + *Ocimum basilicum* (basil)	*P. aeruginosa* (ATCC 27858)	0.63	A	[6]
Lavandula angustifolia (lavender) + *Origanum majorana* (marjoram)	*C. albicans* (ATCC 10231)	0.42	S	[6]
Lavandula angustifolia (lavender) + *Origanum majorana* (marjoram)	*S. aureus* (ATCC 6538)	4.00	An	[6]
Lavandula angustifolia (lavender) + *Origanum majorana* (marjoram)	*P. aeruginosa* (ATCC 27858)	0.52	A	[6]
Lavandula angustifolia (lavender) + *Pelargonium odoratissimum* (geranium)	*C. albicans* (ATCC 10231)	1.04	I	[6]
Lavandula angustifolia (lavender) + *Pelargonium odoratissimum* (geranium)	*S. aureus* (ATCC 6538)	1.17	I	[6]
Lavandula angustifolia (lavender) + *Pelargonium odoratissimum* (geranium)	*P. aeruginosa* (ATCC 27858)	0.52	A	[6]
Lavandula angustifolia (lavender) + *Piper nigrum* (black pepper)	*C. albicans* (ATCC 10231)	0.42	S	[6]
Lavandula angustifolia (lavender) + *Piper nigrum* (black pepper)	*S. aureus* (ATCC 6538)	1.00	I	[6]
Lavandula angustifolia (lavender) + *Piper nigrum* (black pepper)	*P. aeruginosa* (ATCC 27858)	0.57	A	[6]
Lavandula angustifolia (lavender) + *Rosmarinus officinalis* (rosemary)	*C. albicans* (ATCC 10231)	0.42	S	[6]

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Table 9. (cont.)

Essential oil	Pathogen strain	FIC	Result[a]	Reference
Lavandula angustifolia (lavender) + *Rosmarinus officinalis* (rosemary)	*S. aureus* (ATCC 6538)	0.75	A	[6]
Lavandula angustifolia (lavender) + *Rosmarinus officinalis* (rosemary)	*P. aeruginosa* (ATCC 27858)	0.51	A	[6]
Lavandula angustifolia (lavender) + *Salvia sclarea* (sage)	*C. albicans* (ATCC 10231)	0.73	A	[6]
Lavandula angustifolia (lavender) + *Salvia sclarea* (sage)	*S. aureus* (ATCC 6538)	1.00	I	[6]
Lavandula angustifolia (lavender) + *Salvia sclarea* (sage)	*P. aeruginosa* (ATCC 27858)	0.51	A	[6]
Lavandula angustifolia (lavender) + *Santalum album* (sandalwood)	*C. albicans* (ATCC 10231)	0.42	S	[6]
Lavandula angustifolia (lavender) + *Santalum album* (sandalwood)	*S. aureus* (ATCC 6538)	2.25	I	[6]
Lavandula angustifolia (lavender) + *Santalum album* (sandalwood)	*P. aeruginosa* (ATCC 27858)	0.51	A	[6]
Lavandula angustifolia (lavender) + *Styrax benzoin* (benzoin)	*C. albicans* (ATCC 10231)	0.42	S	[6]
Lavandula angustifolia (lavender) + *Styrax benzoin* (benzoin)	*S. aureus* (ATCC 6538)	1.00	I	[6]
Lavandula angustifolia (lavender) + *Styrax benzoin* (benzoin)	*P. aeruginosa* (ATCC 27858)	0.58	A	[6]
Lavandula angustifolia (lavender) + *Thymus vulgaris* (thyme)	*C. albicans* (ATCC 10231)	0.67	A	[6]
Lavandula angustifolia (lavender) + *Thymus vulgaris* (thyme)	*S. aureus* (ATCC 6538)	0.4	S	[6]
Lavandula angustifolia (lavender) + *Thymus vulgaris* (thyme)	*P. aeruginosa* (ATCC 27858)	0.51	A	[6]

[a] S – Synergistic, A – Additive, I – Indifferent/Non-interactive, An – Antagonistic. [b] Value not mentioned.

Essential Oils in Combination for a Holistic Effect

Essential oils when used in combination are not only blended for potential antimicrobial synergy, but also for a presumed holistic synergistic effect. Essential oils have been shown to elicit anti-oxidative (*Table 10*), anti-inflammatory (*Table 11*), as well as antihistaminic effects (*Table 12*), and thus a combination of essential oils may act on varying pathways to treat the respiratory infection on various levels.

Induced oxidant stress is a process that develops when a system undergoes imbalance, such as in infection, resulting in the development of reactive oxygen intermediates (ROI's) and free radicals. The development of ROI's and free radicals leads to cell damage and inflammation and are essential in a hosts defense against infection; however, uncontrolled oxidative reactions are harmful to normal tissues.[247] The respiratory system is particularly susceptible to oxidative stress-mediated injury due to the large surface area associated to gas exchange.[248–250] Oxidative stress-mediated injury includes damage to lung tissue, mucus hypersecretion, edema of the bronchial wall, bronchoconstriction and inflammation.[250–253] Antioxidants are molecules that interact with free radicals causing stabilization within the host and reducing this oxidative stress-mediated damage to healthy tissue. Several studies have shown *in vitro* antioxidant potential of essential oils.[66,119,203–206, 207–218,254] Antioxidant activity *in vitro* is measured by means of several varying test methods. Literature states that conclusive antioxidant activity should not be measured by one method, and as such most literature evaluates the antioxidant activity of test samples using a wide range of methods.[255] Among antioxidant test methodology, the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging method is considered the most regularly applied and reliable method as the process is rapid, simple and inexpensive in comparison to other assays,[255] and hence reported here.

All essential oils prescribed by aroma-therapeutic text for the treatment of respiratory infections show some degree of antioxidant activity by means of DPPH free radical scavenging. The most effective antioxidant activity was identified for *C. zeylanicum* essential oil with 91.4% DPPH inhibition.[206] In recent studies, *C. zeylanicum* has demonstrated the most effective antioxidant activity when compared with other aromatic herbs and spices.[256] Other essential oils that demonstrate antioxidant effects include; *Helichrysum italicum* (immortelle), *Melaleuca cajuputi* (cajuput), *O.*

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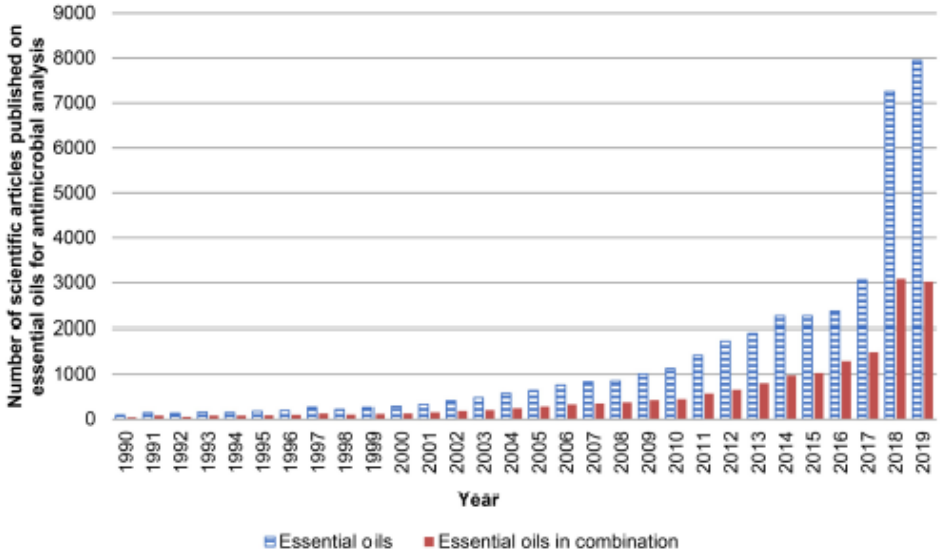


Figure 6. Number of scientific publications on PubMed, ScienceDirect and Scopus pertaining to the antimicrobial properties of essential oils.

basilicum and *O. vulgare* with DPPH inhibition values ranging from 55.7–89.0 %.[206,218–221] From an antimicrobial perspective, the combination of *C. zeylanicum* with *L. angustifolia* essential oil against the pathogen *P. aeruginosa* demonstrated an additive effect (IFIC value of 0.53). When combined, the essential oils may provide an additive antimicrobial effect, while *C. zeylanicum* provides an antioxidant effect, treating the infection from a holistic perspective.

As with the antimicrobial studies performed on essential oils in combination, the available data on combined antioxidant activity of essential oils is not well-documented. It cannot be assumed that the proven antioxidant potential of a single essential oil is maintained when placed in combination and thus the potential for improved antioxidant activity with antimicrobial effects of essential oils in combination needs to be investigated further.

Inflammation is a complex biological response by a host to exposure of infection, damage or chemical changes within a system.[257] In an infection, the body mobilizes the availability of inflammatory markers to increase the presence of immunity modulating cells, such as leukocytes, at the point of infection. This is done to initiate the removal of micro-organisms responsible for infection (*Figure 7*).

Essential oils with anti-inflammatory activity act to reduce swelling and edema associated with respiratory infections, thus reducing symptoms such as wheezing, congestion and difficulty breathing. As with antioxidant activity methods, varying assays are available for the evaluation of anti-inflammatory activity of essential oils. The most commonly employed method is that of the inhibition of edema produced in the hind paw of the rat after injection of a phlogistic agent such as carrageenan or formalin.[258] The carrageenan-induced paw edema study is based on the principle of release of various inflammatory mediators by carrageenan upon injection to tissue, resulting in edema. The inflammatory cells produced by carrageenan include those inflammatory cells associated to respiratory infections. In this method, the animals are dosed with test sample an hour after injection with carrageenan. The degree of edema is compared at various intervals following the introduction of carrageenan, and then compared to a control group. Percentage inhibition of edema is then calculated and used as a measure of anti-inflammatory activity.[258] The effectiveness of essential oils as an anti-inflammatory is directly

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Table 10. The antioxidant activity of essential oils associated to the treatment of respiratory tract infections.

Essential oil Scientific name	Common name	Method (assay)	Solvent	Result	Major chemical constituent	Reference
Allium sativum	Garlic	DPPH scavenging	Methanol	SC_{50}=0.19 μg/ml	diallyl trisulfide (41.62%)	[203]
Allium sativum	Garlic	DPPH scavenging	Methanol	EC_{50}=41±1	n.d.[a]	[204]
Boswellia carterii	Frankincense	DPPH scavenging	Methanol	RC_{50}=266%	*p*-Menth-2-enol (34.5%)	[205]
Canarium luzonicum	Elemi	DPPH scavenging	Ethanol	DPPH inhibition=11.83±0.013%	n.d.	[206]
Carum carvi	Caraway	DPPH scavenging	Methanol	EC_{50}=89±2	n.d.	[204]
Cedrus atlantica	Cedarwood	DPPH scavenging	n.m.[b]	EC_{50}=315.80±0.90	α-Pinene (36.45%)	[185]
Cinnamomum zeylanicum	Cinnamon bark	DPPH scavenging	Ethanol	DPPH inhibition=91.4±0.002%	Cinnamaldehyde (75.32%)	[206]
Cinnamomum zeylanicum	Cinnamon	DPPH scavenging	Methanol	DPPH inhibition=55.3%	Cinnamic aldehyde (67.90%)	[207]
Citrus aurantifolia	Lime	DPPH scavenging	Ethanol	DPPH inhibition=15.22±0.011%	n.d.	[206]
Citrus aurantium	Bitter orange	Conjugated diene	DMSO	AI=9%	Limonene (92.81%)	[208]
Citrus bergamia	Bergamot	Conjugated diene	DMSO	AI=13%	Limonene (38.46%)	[208]
Citrus limon	Lemon	DPPH scavenging	Methanol	RC_{50}=48.90±7.18%	Limonene (64.5%)	[205]
Citrus limon	Lemon	DPPH scavenging	Methanol	EC_{50}=6.90×10^4 g/ml	Limonene (81.4%)	[209]
Citrus sinensis	Orange	DPPH scavenging	Methanol	DPPH inhibition=14.05±0.28%	β-Myrcene, limonene, linalool, decanal and valencene	[210]
Commiphora myrrha	Myrrh	DPPH scavenging	Methanol	AI=12.70±0.42% at concentration 1 mg/ml	n.d.	[211]
Coriandrum sativum	Coriander	Inhibition of 15-LO	Ethanol	IC_{50}=199±11 μg/ml	n.d.	[212]
Cupressus sempervirens	Cypress	DPPH scavenging	Ethanol	IC_{50}=151 μg/ml	α-Pinene (47.51%)	[213]
Cymbopogon citratus	Lemongrass	Thiobarbituric acid reactive species (TBARS)	Methanol	AI=100% at 100 ppm	Geranial (45.7%)	[214]
Cymbopogon martinii	Lemongrass	DPPH scavenging	Methanol	IC_{50}=51.42±1.25	Geraniol (52.49%)	[215]
Daucus carota	Carrot seed	DPPH scavenging	Ethanol	DPPH inhibition=9.01±0.018%	n.d.	[206]
Elettaria cardamomum	Cardamom	DPPH scavenging	Ethanol	DPPH inhibition=23.55±0.015%	n.d.	[206]
Eucalyptus globulus	Eucalyptus	DPPH scavenging	Ethanol	DPPH inhibition=12.53±0.02%	n.d.	[206]
Eugenia caryophyllata	Clove	DPPH scavenging	Methanol	IC_{50}=0.2 μg/ml	Eugenol (88.6%)	[216]
Eugenia caryophyllata	Clove	Conjugated diene	Methanol	AI=52%	Eugenol (76.51%)	[208]
Foeniculum vulgare	Fennel seed	2-hexenal daylight-induced autoxidation	2-hexenal	AI=60% at a concentration of 3.3 μl/ml	Sabinene and 1,8-Cineole	[217]
Helichrysum italicum	Immortelle	DPPH scavenging	DMSO	DPPH inhibition=89%	n.d.	[218]
Hyssopus officinalis	Hyssop	DPPH scavenging	Ethanol	DPPH inhibition=16.48±0.02%	n.d.	[206]

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Table 10. (cont.)

Essential oil Scientific name	Common name	Method (assay)	Solvent	Result	Major chemical constituent	Reference
Laurus nobilis	Bay laurel	DPPH scavenging	Methanol	EC_{50} = 3.9 ± 0.2	n.d.	[204]
Lavandula angustifolia	Lavender	DPPH scavenging	Ethanol	DPPH inhibition = 15.18 ± 0.009 %	n.d.	[206]
Lavandula intermedia	Lavandin	DPPH scavenging	Ethanol	DPPH inhibition = 13.45 ± 0.018 %	n.d.	[206]
Lavandula latifolia	Lavender spike	DPPH scavenging	Ethanol	DPPH inhibition = 10.97 ± 0.015 %	n.d.	[206]
Litsea cubeba	May Chang	DPPH scavenging	Ethanol	DPPH inhibition = 9.05 ± 0.007 %	n.d.	[206]
Melaleuca alternifolia	Tea tree	DPPH scavenging	Methanol	IC_{50} = 29.70 ± 0.70	n.d.	[215]
Melaleuca cajuputi	Cajeput	DPPH scavenging	Methanol	DPPH inhibition = 81 %	Ethanone $C_{16}H_{14}O_4$ (8.81 %)	[219]
Melaleuca quinquenervia	Niaouli	DPPH scavenging	Ethanol	DPPH inhibition = 4.29 ± 0.007 %	n.d.	[206]
Mentha piperita	Peppermint	DPPH scavenging	Methanol	IC_{50} = 860 µg/ml	Menthol (40.7 %)	[220]
Myrtus communis	Myrtle	DPPH scavenging	Methanol	IC_{50} = 5.99 µg/ml	α-Pinene (35.9 %)	[152]
Ocimum basilicum	Basil	DPPH scavenging	Methanol	DPPH inhibition = 55.67 ± 3.38 %	α-Terpineol (59.78 %)	[221]
Origanum majorana	Marjoram	DPPH scavenging	Ethanol	DPPH inhibition = 14.20 ± 0.013 %	n.d.	[206]
Origanum vulgare	Origanum	DPPH scavenging	Ethanol	DPPH inhibition = 86.66 ± 0.008 %	Thymol (83.87 %)	[206]
Pimenta dioica	Pimento	DPPH scavenging	Ethanol	IC_{50} = 0.20 µg/ml	Eugenol (76.00 %)	[157]
Pinus sylvestris	Pine	DPPH scavenging	Ethanol	DPPH inhibition = 31.4 %	n.d.	[222]
Piper nigrum	Black pepper	DPPH scavenging	Methanol	DPPH inhibition = 8 %	Caryophyllene (23.98 %)	[223]
Rosa damascena	Rose	DPPH scavenging	Methanol	IC_{50} = 3.54 ± 1.22 µg/ml	n-Tricosane (16.68 %)	[224]
Rosmarinus officinalis	Rosemary	DPPH scavenging	Methanol	DPPH inhibition = 33.7 %	α-Pinene (14.02 %)	[225]
Salvia officinalis	Sage	DPPH scavenging	Methanol	DPPH inhibition = 9.2 %	1,8-Cineole (28.03 %)	[225]
Syzygium aromaticum	Clove	2-hexenal daylight-induced autooxidation	2-Hexenal	AI = 75.0 % at concentration 3.3 µl/ml	Eugenyl acetate	[217]
Thymus vulgaris	Thyme	DPPH scavenging	Ethanol	DPPH inhibition = 31.62 ± 0.018 %	Linalool (21 %)	[206]
Zingiber officinale	Ginger	DPPH scavenging	Methanol	DPPH inhibition = 36.10 ± 3.51 %	Curcumene (16.67 %)	[221]

[a] Not determined. [b] Not mentioned.

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Table 11. The anti-inflammatory activity of essential oils associated to the treatment of respiratory tract infections.

Essential oil		Method	Result	Major chemical constituent	Reference
Scientific name	Common name				
Allium sativum	Garlic	Carrageenan-induced edema in rats	Edema reduction = 23% at 100 mg/kg	n.d.[a]	[226]
Carum carvi	Caraway	TNF-α suppression assay in mice	TNF-α suppression = 5% at 100 μg/ml	n.d.	[227]
Cinnamomum zeylanicum	Cinnamon	Xylene-induced edema in mice	Edema reduction = 3% at 400 mg/kg	n.d.	[228]
Cinnamomum camphora	Camphor	TNF-α suppression assay	TNF-α suppression = 48.80 ± 0.56% at 100 μg/ml	n.d.	[229]
Citrus aurantifolia	Lime	5-lipoxygenase (5-LOX) inhibition	IC_{50} = 49.35 ppm	Limonene (52.0%)	[209]
Citrus bergamia	Bergamot	Carrageenan-induced edema in rats	Edema reduction = 63.39% at 0.10 μL/kg	n.d.	[230]
Citrus limon	Lemon	5-lipoxygenase (5-LOX) inhibition	IC_{50} = 46.5 ppm	Limonene (81.4%)	[209]
Citrus sinensis	Orange	Carrageenan-induced edema in mice	Edema reduction = notable reduction at 250 mg/kg	n.d.	[231]
Commiphora myrrha	Myrrh	Formalin-induced edema in mice	Edema reduction = 62.8% at 100 mg/kg	n.d.	[232]
Coriandrum sativum	Coriander	Carrageenan-induced edema in rats	Edema reduction = 31% at 100 mg/kg	n.d.	[226]
Cymbopogon citratus	Lemongrass	Carrageenan-induced edema in rats	Edema reduction = 100% at 1.80 mg/kg	Citronellal (83.5%)	[233]
Elettaria cardamomum	Cardamom	Carrageenan-induced edema in rats	Edema reduction = 86.4% at 280 μL/kg	n.d.	[234]
Eucalyptus globulus	Eucalyptus	Carrageenan-induced edema in rats	Edema reduction = 18% at 100 mg/kg	n.d.	[226]
Eugenia caryophyllata	Clove	Carrageenan-induced edema in rats	Edema reduction = 90.15% at 0.05 ml/kg	β-Caryophyllene (44.7%)	[235]
Foeniculum vulgare	Fennel	Carrageenan-induced edema in rats	Edema reduction = 56.78% at 0.20 ml/kg	(E)-Anethole (74.8%)	[236]
Helichrysum italicum	Immortelle	Phospholipase A2 (PLA2)- and serotonin-induced edema in mice	Edema reduction = 65% at 100 mg/kg	n.d.	[218]
Hyssopus officinalis	Hyssop	Carrageenan-induced edema in rats	Edema reduction = 9% at 100 mg/kg	n.d.	[226]
Laurus nobilis	Bay	Carrageenan-induced edema in rats	Edema reduction = 19% at 100 mg/kg	n.d.	[226]
Lavandula angustifolia	Lavender	Carrageenan-induced edema in rats	Edema reduction = 48% at 200 mg/kg	1,8-Cineole (65.4%)	[237]
Lavandula intermedia	Lavandin	5-lipoxygenase (5-LOX) inhibition	Percentage inhibition = 32.6% at 0.30 μL/ml	Linalool (34%)	[238]
Lavandula latifolia	Lavender spike	5-lipoxygenase (5-LOX) inhibition	Percentage inhibition = 38.0% at 0.30 μL/ml	Linalool (47.7%)	[238]
Mentha piperita	Peppermint	Xylene-induced edema in mice	Edema reduction = 50% at 400 mg/kg	n.d.	[228]
Myrtus communis	Myrtle	Carrageenan-induced edema in rats	Edema reduction = 7% at 100 mg/kg	n.d.	[226]
Ocimum basilicum	Basil	Carrageenan-induced edema in mice	Edema reduction = 44% at 50 mg/kg	Estragole (60.96%)	[239]
Origanum vulgare	Origanum	Carrageenan-induced edema in rats	Edema reduction = 16% at 100 mg/kg	n.d.	[226]
Pimenta dioica	Pimento	HRBC membrane stabilization method	64.02% protection of HRBC membrane	n.d.	[240]
Pinus sylvestris	Pine	Acetic acid-induced capillary permeability	Percentage inhibition = 27.7% at 200 mg/ml	n.d.	[241]
Piper nigrum	Black pepper	Carrageenan-induced edema in mice	Edema reduction = 72% at 500 mg/kg	Caryophyllene (23.98%)	[223]
Rosa damascena	Rose	Carrageenan-induced edema in rats	Edema reduction = 30% at 400 μl/kg	n.d.	[242]

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Table 11. (cont.)

Essential oil		Method	Result	Major chemical constituent	Reference
Scientific name	Common name				
Rosmarinus officinalis	Rosemary	Carrageenan-induced edema in rats	Edema reduction at 500 mg/kg	Myrcene (24.6%)	[243]
Salvia officinalis	Sage	Carrageenan-induced pleurisy in rats	Edema reduction = 3.40% at 800 mg/kg	α-Thujone (90%)	[244]
Salvia sclarea	Clary sage	Carrageenan-induced edema in rats	Edema reduction = 70–75 % at 250 mg/kg	Estragole (49%)	[245]
Thymus vulgaris	Thyme	5-lipoxygenase (5-LOX) inhibition	IC_{50} = 0.005 ± 0.00 µl/ml	Thymol (21.46%)	[215]
Zingiber officinale	Ginger	5-lipoxygenase (5-LOX) inhibition	Percentage inhibition = 50.9% at 0.40 mg/ml	Curcumene (16.7%)	[221]

[a] Not determined.

Table 12. The antihistaminic activity of essential oils associated to the treatment of respiratory tract infections.[246]

Essential oil		Result (β-hexosaminidase reduced)
Scientific name	Common name	
Carum carvi	Caraway	20%
Citrus aurantifolia	Lime	0%
Citrus limon	Lemon	35–40%
Citrus sinensis	Orange	10–15%
Cymbopogon citratus	Lemongrass	55%
Daucus carota	Carrot seed	25–30%
Eucalyptus globulus	Eucalyptus	0%
Ocimum basilicum	Basil	30%
Salvia officinalis	Sage	5–10%
Santalum album	Sandalwood	50%
Syzygium aromaticum	Clove	15%
Thymus vulgaris	Thyme	5–10%

correlated to percentage edema reduction. Fifty percent of the essential oils investigated by means of carrageenan-induced paw edema reduction methodology demonstrated anti-inflammatory effect (percentage reductions in edema > 50.0%). In the study, *Eugenia caryophyllata* (clove) demonstrated the highest degree of anti-inflammatory activity with a percentage edema reduction of 90.15%.[235] This outcome is further supported in other essential oil studies[259–261] and augments the prescribed use of essential oils as an anti-inflammatory agent. Other essential oils of significance include *Coriandrum sativum* (coriander, % reduction = 71.7%), *Piper nigrum* (black pepper, % reduction = 72.0%) and *Salvia sclarea* (clary sage, % reduction = 70.0–75.0%).[212,236,246]

As with studies relating to the antioxidant activity of essential oils, it is hypothesized that antimicrobial and anti-inflammatory responses of these agents may be improved in combination, however, this hypothesis requires further investigation as the effects of essential oils in combination for anti-inflammatory purposes is not well documented. Interactions between essential oils therefore may reduce the proven anti-inflammatory activity of a single essential oil when used in combination. Furthermore, emphasis with respect to the anti-inflammatory properties of essential oils in combination for the management of respiratory inflammation should consider assays that target associated inflammatory markers. Inflammatory markers of importance include interleukins (IL-6, 8, 10, 12, 15, 18), TNF-α and γ-interferon.

Histamine is an inflammatory compound that is released from within leukocytes, such as neutrophils

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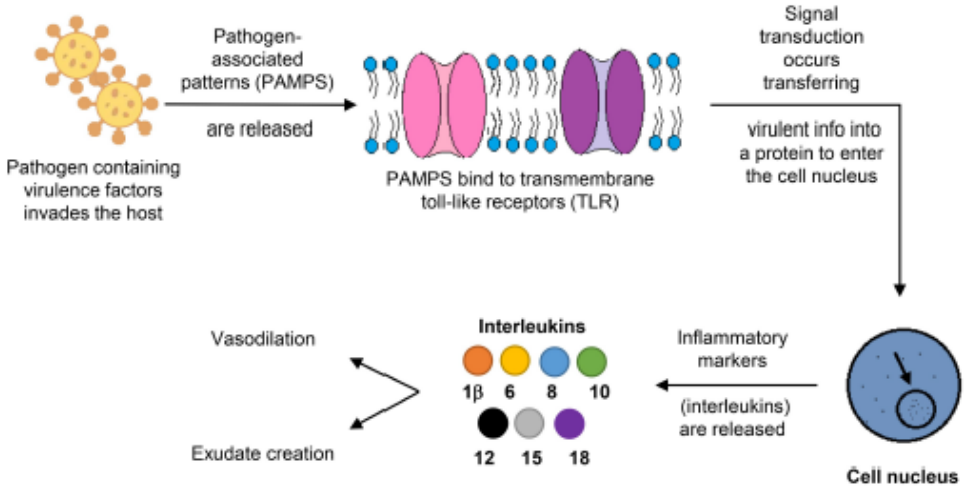


Figure 7. Inflammatory cascade in respiratory infections.

and mast cells, upon exposure to an allergen or antigen. The release of histamine in respiratory tract infections results in bronchoconstriction, vasoconstriction and dilatation, microvascular leak, activation of sensory nerves and increases in mucus secretion.[262–264] These responses by the host cells cause the host to experience symptoms commonly associated with respiratory infections such as watery eyes, nasal congestion and coughs. Essential oils are employed in aroma-therapeutic practice to reduce the edema associated with these symptoms and thus help to clear nasal congestion and aid in symptom relief. The study of essential oils for antihistamine activity has not been well documented with only one scientific publication identified with relevance to respiratory infections.[246] The methodology employed by Mitoshi et al.[246] was that of the β-hexosaminidase release assay. The chemical employed in this assay, β-hexosaminidase, has been indicated in the literature to correlate to histamine release by mast cell and thus used as a measure for antihistamine activity. This assay measures the amount of β-hexosaminidase released from cells following exposure to an antigen and then essential oil. The percentage by which β-hexosaminidase release is reduced is a measure of the effectiveness of essential oil for antihistaminic effects. Of the essential oils investigated, 83.3% of the essential oils showed antihistaminic activity with *C. citratus* demonstrating the highest effect with 55% β-hexosaminidase reduction.[246] Other essential oils with antihistaminic potential include *Santalum album* (sandalwood, β-hexosaminidase reduction = 50%), *C. limon* (β-hexosaminidase reduction = 40%) and *D. carota* (β-hexosaminidase reduction = 30%).[247] *Daucus carota* has proven to elicit noteworthy antimicrobial potential individually as well as in essential oil combinations. It can therefore be hypothesized that *D. carota* in combination with other essential oils for the treatment of respiratory tract infections may produce antimicrobial effects that are supported by antihistaminic and anti-inflammatory effects and therefore, this essential oil in combination should be investigated further.

Essential Oils in Combination with Conventional Antimicrobial Agents

Due to the emergence of growing levels of antimicrobial resistance, the need for greater and more effective antimicrobial drug formulations is fast becoming a matter of urgency. The WHO has stated that 'antibiotic resistance is no longer a prediction for the future; but is happening right now, across the world, and is putting at risk the ability to treat common infections

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in the community and hospitals'.[265] Antimicrobial resistance studies O'Neill,[266] identified that as of 2014, 50 000 deaths within Europe and the United States were attributed to antimicrobial resistance with projections of 10 million deaths globally by 2050. To reduce the burden attributed to antimicrobial resistance, the WHO has created a five-point strategic plan. One of the five points is to optimize the use of current antimicrobial agents.[265] This can be undertaken by combining varying classes of antibiotics with natural products such as essential oils.[267,268] The interest in natural products as a means of combating antimicrobial resistance is steadily on the increase. Studies have shown that essential oils provide an advantage in combating antimicrobial resistance due to their ability to exhibit simultaneous activities toward different bacterial structures.[70,268] This is as a result of the interaction of their multiple compounds as previously mentioned.[269] Combining antimicrobial agents with essential oils provides an opportunity to overcome resistance due to the complexity of the antimicrobial action of these combinations.[268]

Clinical aromatherapy, or the use of essential oils for a targeted clinical symptom, is fast becoming an accepted practice within settings such as hospitals and laboratories. The practice of aromatherapy by nursing staff has been legislated in Minnesota by The Minnesota Board of Nursing, where it is stated that nurses may make use of complementary and integrative care strategies, including aromatherapy, in clinical practice. Furthermore, the practice of clinical aromatherapy has been widely documented and supported by research. In Cape Town (South Africa), the Brooklyn Chest Hospital, makes use of an essential oil mixture comprising of *Melaleuca quinquenervia* (niaouli), *Eucalyptus smithii* (eucalyptus), *Myrtus communis* (myrtle), *Abies balsamea* (fir balsam), *M. alternifolia* (tea tree), *Pelargonium asperum* (geranium) and *M. piperita* essential oils through diffusion among pediatric tuberculosis patients to reduce microbial content in the air.[270]

Albeit that essential oils are employed in clinical settings, studies based on the combination of antibiotics and essential oils are limited, and in some instances irrelevant for practical application, particularly in relation to essential oils and antibiotics used for respiratory infections. *Table 13* summarizes the studies on these interactive combinations performed using microdilution assays. Most of the studies have shown essential oils to enhance antimicrobial activity of antibiotics and antifungals with 82.9% of antimicrobial and essential oil combinations demonstrating favorable interactions. These findings may be as a result of authors merely publishing interactions of favorable outcome and omitting those in relation to antagonistic or indifferent results.

Origanum vulgare essential oil displayed the greatest degree of synergy across both bacterial (IFIC= 0.31) and fungal (IFIC = 0.03) pathogens when combined with gentamicin and amphotericin B against *S. aureus* and *C. albicans*, respectively.[277,281] The investigation of such antimicrobial agents in combination with essential oils is not entirely necessary as the future application of such a combination is not feasible. The antimicrobial agent amphotericin B is an expensive, thermolabile and an intravenous preparation. The application of an essential oil to an intravenous antimicrobial is not achievable. Previous other studies have identified *O. vulgare* essential oil to improve the activity of more practical antimicrobial agents such as β-lactam antibiotics against both Gram-positive and Gram-negative bacteria.[50,272,284] *Pelargonium asperum* essential oil displayed the greatest degree of broad-spectrum activity as synergy was determined for this essential oil in combination with four antimicrobial agents against two pathogens, *S. aureus* (ciprofloxacin, IFIC = 0.50; norfloxacin, IFIC = 0.37 – 0.38) and *C. albicans* (amphotericin B, IFIC = 0.04; ketoconazole, IFIC = 0.25). Fungal pathogens (when compared to bacterial pathogens) demonstrated the greatest degree of sensitivity to the effects of essential oil-conventional antimicrobial combinations. This is in alignment with literature in which studies have proven that due to cell structure, fungi are more sensitive to the antimicrobial effects of lipids found within natural products such as essential oils.[6,282–283,278]

The use of essential oils as conjugates to antimicrobial therapy is an important consideration within antimicrobial studies as a large population of the world make use of CAM therapies in combination with allopathic treatments.[285] The WHO has emphasized the urgent need to closely integrate the use of CAM treatments among allopathic practices in order to improve on patient care and treatment outcomes.[285] These practices can only be integrated on the proven potential of these products in combined use and therefore the antimicrobial activity of essential oils in combination with conventional antimicrobial agents used for the treatment of respiratory related infections should be encouraged to meet those standards set by the WHO.

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Table 13. Antibiotic/essential oil combination studies against pathogens associated with respiratory tract infections.

Antimicrobial / Essential oil combination		FIC	Result[a]	Reference
Staphylococcus aureus				
Amoxicillin	*Elettaria cardamomum* (cardamom)	1.06	I	[271]
Amoxicillin	*Origanum vulgare* (origanum)	0.75	A	[272]
Chloramphenicol	*Lavandula angustifolia* (lavender)	0.75	A	[273]
Ciprofloxacin	*Elettaria cardamomum* (cardamom)	0.62	A	[271]
Ciprofloxacin	*Melaleuca alternifolia* (tea tree)	7.70	An	[70]
Ciprofloxacin	*Mentha piperita* (peppermint)	0.75	A	[70]
Ciprofloxacin	*Pelargonium graveolens* (geranium)	0.375	S	[274]
Ciprofloxacin	*Rosmarinus officinalis* (rosemary)	1.03	A	[70]
Ciprofloxacin	*Thymus vulgaris* (thyme)	0.80	A	[70]
Ciprofloxacin	*Lavandula angustifolia* (lavender)	0.49	S	[273]
Fusidic acid	*Lavandula angustifolia* (lavender)	0.65	A	[273]
Gentamicin	*Melaleuca alternifolia* (tea tree)	0.49	S	[275]
Gentamicin	*Origanum vulgare* (origanum)	0.31	S	[276]
Norfloxacin	*Pelargonium graveolens* (geranium)	0.37	S	[277]
Norfloxacin	*Pelargonium graveolens* (geranium)	0.38	S	[277]
Vancomycin	*Melaleuca alternifolia* (tea tree)	>0.50	A	[275]
Streptococcus pneumoniae				
Amoxicillin	*Illicium verum* (star anise)	2.00	I	[278]
Amoxicillin	*Illicium verum* (star anise)	1.03	I	[278]
Amoxicillin	*Illicium verum* (star anise)	0.50	S	[278]
Ciprofloxacin	*Illicium verum* (star anise)	2.00	I	[278]
Ciprofloxacin	*Illicium verum* (star anise)	1.06	I	[278]
Ciprofloxacin	*Illicium verum* (star anise)	0.62	A	[278]
Klebsiella pneumoniae				
Cefixime	*Thymus vulgaris* (thyme)	1.00	A	[279]
Ciprofloxacin	*Melaleuca alternifolia* (tea tree)	0.73	A	[70]
Ciprofloxacin	*Mentha piperita* (peppermint)	0.68	A	[70]
Ciprofloxacin	*Pelargonium graveolens* (geranium)	0.375	S	[274]
Ciprofloxacin	*Rosmarinus officinalis* (rosemary)	0.28	S	[70]
Ciprofloxacin	*Thymus vulgaris* (thyme)	0.71	A	[70]
Ciprofloxacin	*Thymus vulgaris* (thyme)	0.37	S	[279]
Gentamicin	*Thymus vulgaris* (thyme)	0.50	S	[279]
Pseudomonas aeruginosa				
Chloramphenicol	*Lavandula angustifolia* (lavender)	0.29	S	[273]
Ciprofloxacin	*Ocimum basilicum* (basil)	1.03	I	[280]
Ciprofloxacin	*Lavandula angustifolia* (lavender)	0.74	A	[273]
Fusidic acid	*Lavandula angustifolia* (lavender)	1.13	N	[273]
Imipenem	*Ocimum basilicum* (basil)	0.75	A	[280]
Candida albicans				
Amphotericin B	*Pelargonium graveolens* (geranium)	0.04	S	[281]
Amphotericin B	*Origanum vulgare* (origanum)	0.03	S	[281]
Amphotericin B	*Melaleuca alternifolia* (tea tree)	0.13	S	[281]
Amphotericin B	*Thymus vulgaris* (thyme)	n.m.[b]	S	[282]
Chloramphenicol	*Lavandula angustifolia* (lavender)	1.00	A	[273]
Fluconazole	*Ocimum sanctum* (basil)	0.24 – 0.63	S	[283]
Ketoconazole	*Ocimum sanctum* (basil)	0.41 – 0.52	S	[283]
Ketoconazole	*Pelargonium asperum* (geranium)	0.25	S	[283]
Nystatin	*Lavandula angustifolia* (lavender)	1.14	I	[273]

[a] S – Synergistic, A – Additive, I – Indifferent/Non-interactive, An – Antagonistic. [b] Value not mentioned.

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Allopathic Use of Essential Oils

The antimicrobial and therapeutic effects of essential oils proven individually have been widely accepted by the pharmaceutical industry. Several essential oil-containing products are currently sold on the market with emphasis on the ability of these products to treat and aid in symptomatic management. The most commonly employed product is that of Vicks VapoRub®, a formulation containing essential oils of camphor, eucalyptus oil and menthol. Vicks VapoRub® has been marketed since 1894 and has been a global household staple for over a century in the management and treatment of respiratory infections. A clinical study conducted by Paul et al.[286] aimed to identify the effects of Vicks VapoRub® when compared to a placebo on the symptoms associated with respiratory infections. The trial consisted of 138 children, each suffering from a respiratory tract infection causing cough and congestion. By night two of the trial, Vicks VapoRub® had substantially reduced the symptoms of cough and congestion while aiding in patient sleep and recovery.[287] Pinimenthol®, an ointment comprising of eucalyptus, pine-needle and menthol essential oils, is another product marketed for the treatment of respiratory tract infections and related symptoms. Post-marketing studies on the use of this product identified that 88.3% of patients found the product to be tolerable with 41.4% of patients demonstrating complete resolve of symptoms after 5 to 14 days.[288]

Menthol, however, although applied in pharmaceutical products, has poor activity in the treatment of respiratory infections and symptoms. Studies conducted as early as 1943 identified that the inhalation of menthol produced an increase in nasal resistance within one minute after administration. This nasal congestion was further sustained for a period exceeding 210 minutes. Patients having received this treatment, however, noted that their nasal passages 'felt clear' despite the increase in congestion.[289,290] Further studies have highlighted the respiratory depression, increased mucous production and decreased mucociliary beat associated with low doses of menthol.[291–297]

These adverse effects demonstrate the importance of scientific validation to confirm product efficacy and safety, as it cannot be assumed that good or significant *in vitro* activity will translate as such *in vivo*. In addition, essential oils and essential oil products are not regulated. Due to the lack in regulation of these products, consumers are not adequately informed of the indications, directions for use and safety data resulting in misuse of these products.[298]

Toxicity of Essential Oils

Essential oils have been associated with the highest number of toxicities when compared to other natural products. This is because of the high concentration of essential oils formulated within products which require small doses to elicit a toxic effect.[298] The majority of cases involving toxicity or poisoning, are predominantly associated with accidental ingestion by children.[299]

A summary of the known toxicity profiles of essential oils indicated for use in respiratory infections is given in *Table 14*.

The acute oral toxicity values have been cited for these essential oils as acute inhalation toxicity has not been well reported. Acute inhalation toxicity is primarily qualitative in measurement as calculation of the quantity of inhaled volatile resulting in toxicity is difficult to measure.[299] Acute oral toxicity is expressed as an LD_{50} value, or the dose responsible for a mortality of 50.0% of tested animal subjects. Toxicologists deem a test substance to be extremely toxic if an LD_{50} value of 9.00 mg/kg or less is reported. A product or substance is considered very toxic if an LD_{50} value of between 10.00 and 99.00 mg/kg is determined, moderately toxic for an LD_{50} value of 100.00–999.00 mg/kg and slightly toxic if the LD_{50} value is between 1.00 and 5.00 g/kg. A product or substance is only considered to be safe or non-toxic for an LD_{50} value of >5.00 g/kg.[314] Of the essential oils reviewed, 43.8% are considered non-toxic while the remaining essential oils are considered slightly to moderately toxic (43.7%) or unknown for toxicity (12.5%).

Interestingly, some essential oils for example, *Cinnamomum camphora* (camphor) and *M. piperita*, most commonly employed within pharmaceutical preparations for an assumed antimicrobial effect in respiratory infections, were determined as moderately toxic with LD_{50} values of 0.80 g/kg and 0.82 g/kg, respectively. Studies have highlighted that the toxicity experienced by these oils is predominantly due to their chemistry.[315,316] *Table 15* further demonstrates the toxicity associated to the chemistry of essential oils.

The major chemical compounds of *C. camphora* and *M. piperita*, being camphor and menthol respectively, produce an average LD_{50} value of 0.80 g/kg and 0.10 g/kg, respectively. Toxicity symptoms include increase in respiratory rate and osmotic swelling, indicating damage to biological tissue once inhaled.[323]

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Table 14. The toxicity of essential oils associated to the treatment of respiratory tract infections.

Essential oil Scientific name	Common name	Adverse reactions	Acute toxicity	Result[a]	Regulatory authority[b]	Reference
Abies balsamea	Fir	Skin sensitivity	LD_{50} = unknown	n.d.	IFRA regulated	[300]
Allium sativum	Garlic	Skin sensitivity, hypotension, anticoagulation	LD_{50} = > 5.00 g/kg	not toxic	Not regulated	[301,302]
Amyris balsamifera	Amyris	None known	LD_{50} = > 5.00 g/kg	not toxic	Not regulated	[303]
Boswellia carterii	Frankincense	Skin sensitivity	LD_{50} = > 5.00 g/kg	not toxic	Not regulated	[304]
Canarium luzonicum	Elemi	None known	LD_{50} = 3.37 g/kg	slightly toxic	IFRA regulated	[304,305]
Carum carvi	Caraway	None known	LD_{50} = 3.50 ml/kg	slightly toxic	GRAS status, IFRA regulated	[304,305]
Cedrus atlantica	Cedarwood	None known	LD_{50} = > 5.00 g/kg	not toxic	Not regulated	[304]
Cinnamomum camphora	Camphor	None known	LD_{50} = 5.10 ml/kg	not toxic	IFRA regulated	[306]
Cinnamomum zeylanicum	Cinnamon	Skin sensitivity	LD_{50} = 3.40 ml/kg	slightly toxic	GRAS status, IFRA and EU regulated	[304]
Citrus aurantifolia	Lime	Phototoxic	LD_{50} = > 5.00 g/kg	not toxic	GRAS status, IFRA and SCCNFP regulated	[304]
Citrus bergamia	Bergamot	None known	LD_{50} = > 5.00 g/kg	not toxic	IFRA and SCCNFP regulated	[304]
Citrus limon	Lemon	Phototoxic	LD_{50} = > 5.00 g/kg, ED_{50} = 6.00 g/kg	not toxic	IFRA and SCCNFP regulated	[307]
Citrus sinensis	Orange	Skin sensitivity	LD_{50} = > 5.00 g/kg	not toxic	GRAS status, IFRA regulated	[299,307]
Commiphora myrrha	Myrrh	Fetotoxic	LD_{50} = 1.65 g/kg	slightly toxic	Not regulated	[308]
Coriandrum sativum	Coriander	None known	LD_{50} = > 5.00 g/kg	not toxic	GRAS status, IFRA regulated	[306]
Cupressus sempervirens	Cypress	Skin sensitivity	LD_{50} = > 5.00 g/kg	not toxic	Not regulated	[299]
Cymbopogon citratus	Lemongrass	Skin sensitivity, teratogenic, anticoagulation	LD_{50} = > 5.00 g/kg	not toxic	GRAS status, IFRA regulated	[308]
Daucus carota	Carrot seed	Antigestational	LD_{50} = > 5.00 g/kg	not toxic	GRAS status	[308]
Elettaria cardamomum	Cardamom	Neurotoxic, labors breathing in children	LD_{50} = 5.00 g/kg	slightly toxic	GRAS status	[307]
Eucalyptus citriodora	Eucalyptus	Neurotoxic, labors breathing in children	LD_{50} = 3.70 ml/kg	slightly toxic	Canada (Health Canada Cosmetic Ingredient Hotlist), Australia (Schedule 6 poison)	[309]
Ferula galbaniflua	Galbanum	Skin sensitivity	LD_{50} = > 5.00 g/kg	not toxic	Not regulated	[299]

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Table 14. (cont.)

Essential oil Scientific name	Common name	Adverse reactions	Acute toxicity	Result[a]	Regulatory authority[b]	Reference
Foeniculum vulgare	Fennel	Skin sensitivity, estrogenic, cardnogenic, anticoagulation, glucose intolerance	LD_{50} = 1.33 g/kg, max. adult dose: 73.00 mg daily	slightly toxic	IFRA and EU regulated	[310]
Helichrysum angustifolium	Immortelle	Skin sensitivity	LD_{50} = 4.40 g/kg	slightly toxic	GRAS status	[311]
Hyssopus officinalis	Hyssop	None known	LD_{50} = not known	n.d.	IFRA regulated	[299]
Laurus nobilis	Bay	Skin sensitivity	LD_{50} = not known	n.d.	EU regulated, Canada (Health Canada Cosmetic Ingredient Hotlist)	[299]
Lavandula angustifolia	Lavender	None known	LD_{50} = 4.25 g/kg	slightly toxic	GRAS status, IFRA regulated	[308]
Lavandula burnati	Lavandin	Skin sensitivity	LD_{50} = > 5.00 g/kg	not toxic	GRAS status, IFRA regulated	[312]
Lavandula latifolia ci neolifera	Lavender spike	Neurotoxic	LD_{50} = 3.80 g/kg	slightly toxic	GRAS status, IFRA regulated	[308]
Melaleuca alternifolia	Tea tree	Skin sensitivity	LD_{50} = 1.90 g/kg	slightly toxic	BiR, IFRA and EU regulated, Australia (Schedule 6 poison)	[312]
Melaleuca cajuput	Cajeput	Neurotoxic, labors breathing in children	LD_{50} = 3.87 g/kg	slightly toxic	Not regulated	[303]
Melaleuca viridiflora	Niaouli	None known	LD_{50} = not known	n.d.	Not regulated	[299]
Mentha piperita	Peppermint	Neurotoxic, mucous membrane sensitivity, liver toxicity, antkoagulation	LD_{50} = 0.82 g/kg	moderately toxic	GRAS status	[299]
Myrtus communis	Myrtle	Anticoagulation, glucose intolerance	LD_{50} = > 5.00 g/kg	not toxic	IFRA and EU regulated	[313]
Ocimum basilicum	Basil	Skin sensitivity	LD_{50} = not known	n.d.	IFRA and EU regulated	[299]
Origanum majorana	Marjoram	None known	LD_{50} = 2.24 g/kg	slightly toxic	GRAS status	[308]
Origanum vulgare	Origanum	Skin sensitivity, mucous membrane sensitivity, embryo toxic, anticoagulation	LD_{50} = 1.85 g/kg	slightly toxic	GRAS status	[307]
Pimenta officinalis	Pimento	Skin sensitivity, anticoagulation, mucous membrane sensitivity	LD_{50} = 3.60 ml/kg	slightly toxic	IFRA and EU regulated	[307]
Pinus sylvestris	Pine	Skin sensitivity	LD_{50} = > 5.00 g/kg	not toxic	IFRA and SCCNFP regulated	[308]
Piper nigrum	Black Pepper	Skin sensitivity	LD_{50} = > 5.00 g/kg	not toxic	GRAS status, IFRA regulated	[299]
Rosa × damascena	Rose	None known	LD_{50} = > 5.00 g/kg	not toxic	GRAS status, IFRA, EU and SCCNFP regulated	[304]
Rosmarinus officinalis	Rosemary	Neurotoxic, labors breathing in children	LD_{50} = 5.00 ml/kg	slightly toxic	GRAS status	[307]
Salvia officinalis	Sage	Neurotoxic	LD_{50} = 2.60 g/kg	slightly toxic	EU regulated	[307]

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Table 14. (cont.)

Essential oil Scientific name	Common name	Adverse reactions	Acute toxicity	Result[a]	Regulatory authority[b]	Reference
Salvia sclarea	Clary sage	Skin sensitivity	$LD_{50} = > 5.00$ g/kg	not toxic	GRAS status	[307]
Santalum album	Sandalwood	Skin sensitivity	$LD_{50} = > 5.00$ g/kg	not toxic	Not regulated	[307]
Styrax benzoin	Benzoin	Skin sensitivity	$LD_{50} = 0.37$ g/kg	moderately toxic	Not regulated	[299]
Syzygium aromaticum	Clove	Skin sensitivity, mucous membrane sensitivity, embryo toxic, anticoagulation	$LD_{50} = 2.65$ g/kg	slightly toxic	IFRA, EU and SCCNFP regulated	[304]
Thymus vulgaris	Thyme	Anticoagulation, skin sensitivity, mucous membrane sensitivity	$LD_{50} =$ not known	n.d.	GRAS status, IFRA regulated	[299]
Zingiber officinale	Ginger	None known	$LD_{50} = > 5.00$ g/kg	not toxic	GRAS status	[307]

[a] Extremely toxic – $LD_{50} \leq 9.00$ mg/kg, very toxic – LD_{50} is 10.00–99.00 mg/kg, moderately toxic – LD_{50} is 100.00–999.00 mg/kg, slightly toxic – LD_{50} is 1.00–5.00 g/kg, non-toxic – $LD_{50} > 5.00$ g/kg and n.d. – not determined. [b] IFRA – International Fragrance Association; GRAS – generally recognized as safe; EU – European Union, SCCNFP – Scientific committee on cosmetic products and non-food products; BfR – The German federal institute for risk assessment.

The United States Food and Drug Authority (US, FDA) aims to protect the public through the registration and classification of essential oils for human use. Many essential oils have been registered and classified with the US FDA as 'Generally recognized as safe' (GRAS) products (*Table 14*). Menthol is registered as a GRAS product by the US FDA, with over the counter products containing 16.0% menthol concentrations approved for human use.[324,325] This status, however, is for external application of this product only as usually applicable in the pharmaceutical market. Due to the lack of data regarding the safety of these products for respiratory purposes, misuse of these ingredients by consumers occurs, resulting in adverse and toxic effects. Studies have shown that the direct application of Vicks VapoRub® to the inner nostril of patients has resulted in the development of lipoid pneumonia.[326,327] Lipoid pneumonia is the accumulation of lipid products within the bronchial tree resulting in an inflammatory response by host tissue. Cases of this condition range from asymptomatic to life-threatening.[328–330]

Researchers have further determined that essential oils rich in 1,8-cineole content such as, *Elettaria cardamomum* (cardamom), *R. officinalis*, *E. globulus*, *Lavandula latifolia* (lavender spike) and *M. cajuputi* (cajuput), should not be used in children under the age of ten due to their apneic effects.[299] Further studies have identified the benefit of 1,8-cineole for the treatment of respiratory conditions such as asthma. In a double-blind, placebo-controlled study where patients with asthma were administered with 200 mg/day capsules of 1,8-cineole, the findings demonstrated anti-inflammatory effects on airways due to the inhibition of cytokines along the arachidonic acid pathway, thus resulting in a reduction in corticosteroid therapy.[331–334]

Essential oils and their associated compounds are registered on the GRAS list as individual agents and not as combinations, which are the more frequently applied practice in aromatherapy. The activity between blended molecules has the potential to increase side effects and promote toxicity or quench (purpose of suppressing the harmful effects) the toxicity of major compounds. An example of this phenomenon is highlighted in the combination of *C. citratus* and *Citrus paradisi* (grapefruit) essential oils. Citral, the main chemical compound within *C. citratus* and other citrus-based essential oils has been identified as causing dermal irritation when applied topically. When the two essential oils are combined, the major chemical constituent (limonene) of grapefruit essential oil,

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Table 15. The toxicity of major chemical constituents of essential oils indicated in treatment of respiratory infections.

Essential oil compound	Prevalent in essential oil Scientific name	Common name	Acute toxicity	Result[a]	Reference
Benzyl benzoate	*Styrax benzoin*	Benzoin	LD_{50}=1.90–2.80 g/kg	slightly toxic	[317]
Camphor	*Salvia officinalis*	Sage	LD_{50}=1.31g/kg	slightly toxic	[299]
Carotol	*Daucus carota*	Carrot seed	LD_{50}=unknown	slightly toxic	[299]
Carvacrol	*Origanum vulgare*	Origanum	LD_{50}=0.81g/kg	moderately toxic	[303]
Carvone	*Carum carvi*	Caraway	LD_{50}=1.60 g/kg	slightly toxic	[303]
β-Caryophyllene	*Piper nigrum*	Black pepper	LD_{50}=>5.00 g/kg	not toxic	[306]
1,8-Cineole	*Elettaria cardamomum*	Cardamom	LD_{50}=2.50 g/kg	slightly toxic	[303]
	Rosmarinus officinalis	Rosemary			
	Eucalyptus globulus	Eucalyptus			
	Lavandula latifolia	Lavender spike			
	Melaleuca cajuputi	Cajeput			
Citronellal	*Rosa x damascena*	Rose	LD_{50}=>5.00 g/kg	not toxic	[304]
Diallyl disulfide	*Allium sativum*	Garlic	LD_{50}=0.14 g/kg	moderately toxic	[318]
Diallyl trisulfide	*Allium sativum*	Garlic	LD_{50}=0.08 g/kg	very toxic	[319]
Estragole	*Ocimum basilicum*	Basil	LD_{50}=1.25 g/kg	slightly toxic	[308]
Eugenol	*Cinnamomum zeylanicum*	Cinnamon	LD_{50}=2.68 g/kg	slightly toxic	[303]
	Pimenta dioica	Pimento			
	Syzygium aromaticum	Clove			
Furanoeudesma-1,3-diene	*Commiphora myrrha*	Myrrh	LD_{50}=unknown	n.d.	[299]
Geranial	*Cymbopogon citratus*	Lemongrass	LD_{50}=3.60 g/kg	slightly toxic	[303]
Himachalene	*Cedrus atlantica*	Cedarwood	LD_{50}=unknown	n.d.	[299]
Limonene	*Canarium luzonicum*	Elemi	LD_{50}=>5.00 g/kg	not toxic	[304]
	Citrus aurantifolia	Lime			
	Carum carvi	Caraway			
	Cinnamomum camphora	Camphor			
	Citrus bergamia	Bergamot			
	Citrus limon	Lemon			
	Citrus sinensis	Orange			
Linalool	*Coriandrum sativum*	Coriander	LD_{50}=2.79 g/kg	slightly toxic	[303]
	Ocimum basilicum	Basil			
	Hyssopus officinalis	Hyssop			
	Lavandula intermedia	Lavandula			
Linalyl acetate	*Salvia sclarea*	Clary sage	LD_{50}=>5.00 g/kg	not toxic	[320]
	Lavandula angustifolia	Lavender			
Menthol	*Mentha piperita*	Peppermint	LD_{50}=3.30 g/kg	slightly toxic	[308]
Myrcene	*Rosmarinus officinalis*	Rosemary	LD_{50}=>5.00 g/kg	not toxic	[308]
β-Ocimene	*Laurus nobilis*	Bay	LD_{50}=>5.00 g/kg	not toxic	[299]
α-Pinene	*Abies balsamea*	Fir	LD_{50}=2.10–3.70 g/kg	slightly toxic	[299]
	Helichrysum italicum	Immortelle			
	Boswellia carterii	Frankincense			
	Cupressus sempervirens	Cypress			
	Pinus sylvestris	Pine			

Table 15. (cont.)

Essential oil compound	Prevalent in essential oil Scientific name	Common name	Acute toxicity	Result[a]	Reference
β-Pinene	*Ferula galbaniflua*	Galbanum	$LD_{50} = > 5.00$ g/kg	not toxic	[299]
α-Santalol	*Santalum album*	Sandalwood	$LD_{50} = 3.80$ g/kg	slightly toxic	[321]
Terpinen-4-ol	*Melaleuca alternifolia*	Tea tree	$LD_{50} = 1.30$ g/kg	slightly toxic	[313]
	Origanum majorana	Marjoram			
Terpineol	*Ocimum basilicum*	Basil	$LD_{50} = 4.30$ g/kg	slightly toxic	[308]
Thujone	*Salvia officinalis*	Sage	$LD_{50} = 0.25$ g/kg	moderately toxic	[322]
Thymol	*Thymus vulgaris*	Thyme	$LD_{50} = 0.98$ g/kg	moderately toxic	[303]
trans-Anethole	*Foeniculum vulgare*	Fennel	$LD_{50} = 2.09–3.20$ g/kg	slightly toxic	[303]
Valerianol	*Amyris balsamifera*	Fir balsam	$LD_{50} = > 5.00$ g/kg	not toxic	[303]
Viridiflorol	*Melaleuca viridiflora*	Niaouli	$LD_{50} =$ unknown	n.d.	[299]
Zingiberene	*Zingiber officinale*	Ginger	$LD_{50} = 2.50$ g/kg	slightly toxic	[303]

[a] Extremely toxic – $LD_{50} \leq 9.00$ mg/kg, very toxic – LD_{50} is 10.00 – 99.00 mg/kg, moderately toxic – LD_{50} is 100.00 – 999.00 mg/kg, slightly toxic – LD_{50} is 1.00 – 5.00 g/kg, non-toxic – $LD_{50} > 5.00$ g/kg and n.d. – not determined.

reduces or 'quenches' this toxic effect, rendering the combination safe for use.[6,7] There is limited data on the effect of toxicity of multiple essential oil combinations, specifically essential oils for the treatment of respiratory infections. Studies on the cytotoxic effects of essential oils in combination with carrier oils has been demonstrated with specific interest for dermal application. The study by Orchard et al.[335] demonstrated that several essential oils initially found to display high levels of cytotoxicity, experienced quenching cytotoxic effects with only half or less of the carrier oils combined. These findings demonstrate the potential for essential oils to be used in combination for improved cytotoxic effects. It is therefore suggested that greater attention be given to the toxicity of combined essential oils. Due to the large vascularity and surface area for absorption provided by the lungs, it is of importance to know the risks associated with direct inhalation of essential oils or their constituents. Further to this, the impact of essential oils or their major chemical constituents in combination on inhalation toxicity should also be considered.

Summary of Future Recommendations and Final Conclusions

Of the respiratory infection causing pathogens studied, yeasts and fungi such as *C. albicans* and *C. neoformans*, were found to be the most sensitive to essential oil inhibition, followed by Gram-positive bacteria. Gram-negative bacteria are inherently more resistant to the antimicrobial effects of essential oils due to the complexity of the cell wall. The most frequently studied organisms are *S. aureus*, *K. pneumoniae* and *C. albicans*. The pathogens most commonly isolated in respiratory tract infections are, however, often neglected. These pathogens include *S. pneumoniae*, *S. agalactiae*, *S. pyogenes*, *S. mitis*, *H. influenzae*, *M. tuberculosis*, *C. diphtheriae*, *M. catarrhalis* and *L. pneumophilia*. Furthermore, antiviral studies on the use of essential oils for respiratory infections is severely lacking within the literature with almost no studies available on the effect of these products on viral strains. As respiratory infections are primarily viral in origin, antiviral studies should be considered for future study. In addition, the published data on essential oil antimicrobial efficacy is limited, as researchers continue to make use of the disk diffusion assay for antimicrobial analysis. Due to the inaccuracies associated with disk diffusion assays, it is further

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suggested that studies previously undertaken on the antimicrobial activity of commercial essential oils against respiratory pathogens by means of disk diffusion be reproduced using more reliable testing methodology such as broth microdilution assays.

Essential oil use within aromatherapy is predominantly encouraged in combination; however, the validity of these combinations is often restricted to anecdotal evidence and recommendations made in published books about aromatherapy, and not proven in peer-reviewed journals. The use of essential oils in combination for antimicrobial, antioxidant, anti-inflammatory or antihistaminic effect have not been well-established within the scientific literature. Furthermore, the use of essential oils in combination against respiratory tract pathogens have not been clinically proven within structured clinical trial studies. It is suggested that emphasis be placed on investigating the use of essential oils in combination rather than identifying the potential of essential oils individually. When reviewing the literature, it was determined that less than half of all essential oil studies published are conducted on essential oils in combination. This lack of literature may be due to under-reporting of interactions studied due to the combination of essential oils not revealing what is perceived as a positive effect. Any interaction is a valuable result, be it synergistic, additive, indifferent or antagonistic. Under-reporting of antagonistic interactions runs the risk of ongoing use and potential for harm and resistance. Furthermore, proof of positive interactions may help in the incorporation of such complementary medicines within allopathic practice. Due to the practice of essential oil blending in aroma-therapeutic practice, it is essential that antimicrobial combination studies be conducted to validate use. Essential oil use for antimicrobial purposes and symptomatic management are predominantly cited using layman publications and the internet as resources. It is encouraged that more research is to be undertaken to prove or disprove these claims.

Toxicity studies on the use of essential oils are inadequate with current emphasis on oral toxicity and the use of essential oils on their own. It is recommended that toxicity studies be incorporated to include human cell lines using MTT assays to determine the cytotoxicity of essential oils on human lung tissue. The toxicological effects of essential oils are important facets that need to be addressed.[50] Without the safety profile determination of essential oils, these products cannot be recommended for human use.

In addition, the role of essential oil chemistry in perceived essential oil and essential oil combination activity should be considered for future emphasis. The impact of essential oil chemistry using an unbiased approach such as chemometrics or computational modelling will aid in the predictive potential for essential oil blending. Finally, additional assays such as mechanism of action studies and antioxidant and anti-inflammatory assays should be considered to determine the feasibility of essential oil combinations that prove additive or indifferent antimicrobial effects.

List of abbreviations

CAM	Complementary and Alternative Medicines
DPPH	1,1-Diphenyl-2-picrylhydrazyl
FDA	The United States Food and Drug Authority
FIRS	The Forum of International Respiratory Societies
GRAS	Generally recognized as safe
HRV	Human rhinovirus
IC_{50}	The half maximal inhibitory concentration
IFIC	Fractional Inhibitory Index
ISO	International Standard Organization
LRTI	Lower respiratory tract infections
MIC	Minimum inhibitory concentration
ROIs	Reactive oxygen intermediates
RSV	Respiratory Syncytial Virus
SI	Selective index
TB	Tuberculosis
URTI	Upper respiratory tract infections
WHO	World Health Organization

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CHAPTER 2:
INVESTIGATION OF ESSENTIAL OIL ANTIMICROBIAL ACTIVITY AND CHEMOMETRIC ANALYSIS



2. Introduction

Chapter 1 provided a review of the layman aroma-therapeutic literature and available scientific studies regarding the holistic activity of commercial essential oils indicated for use in the respiratory tract. This chapter focuses on the selection and procurement of the essential oils chosen for analysis, as well as the motivation for pathogens selected for the study. Furthermore, this chapter then considers the chemical and antimicrobial analysis of the selected commercial essential oils.

2.1. Essential oil selection, procurement and storage

A selection of 49 of the 55 previously identified essential oils were obtained from international fragrance companies Robertet© (Grasse, France), Burgess and Finch (Cape Town, South Africa), Prana Monde (Hainaut, Belgium), Scatters Oils (Gauteng, South Africa) and Escentia Oils (Gauteng, South Africa). The essential oils were selected based on the frequency of citation in the aroma-therapeutic literature available to the layman, with specified recommendation in treating respiratory tract infections (Lawless, 1995, Shealy, 1998, Hili, 2001, Sellar, 2005, Clarke, 2009, Buckle, 2014, Curtis et al., 2016). Table 2.1 summarizes the essential oils used in this study. Six of the 55 essential oils previously identified in Chapter 1 were not considered for study due to lack of availability.

Table 2.1: Summary of suppliers from which the essential oils selected for analysis were procured

Scientific name	Common name	Supplier of essential oil
Abies balsamea	Fir	Prana Monde (Hainaut, Belgium)
Amyris balsamifera	Amyris	
Boswellia carteri	Frankincense	
Canarium luzonicum	Elemi	
Carum carvi	Caraway	
Cinnamomum camphora	Camphor	
Cinnamomum zeylanicum	Ceylon cinnamon	
Citrus aurantifolia	Lime	Escentia Oils (Gauteng, South Africa)
Citrus bergamia	Bergamot	Prana Monde (Hainaut, Belgium)
Citrus limon	Lemon	
Citrus sinensis	Orange	
Commiphora molmol	Myrrh	
Coriandrum sativum	Coriander	

Scientific name	Common name	Supplier of essential oil
Cupressus sempervirens	Cypress	
Cymbopogon citratus	Lemongrass	Prana Monde (Hainaut, Belgium)
Daucus carota	Carrot seed	
Elettaria cardamonum	Cardamom	
Eucalyptus globulus	Eucalyptus	
Ferula galbaniflua	Galbanum	Escentia Oils (Gauteng, South Africa)
Foeniculum vulgare	Fennel	Prana Monde (Hainaut, Belgium)
Helichrysum italicum	Imortelle	
Hyssopus officinalis	Hyssop	
Illicium verum	Star-anise	
Juniperus virginiana	Cedarwood	
Laurus nobilis	Bay	
Lavandula angustifolia	Lavender	
Lavandula burnati	Lavandin	
Lavandula spica	Lavender spike	
Melaleuca alternifolia	Tea tree	
Melaleuca cajeput	Cajeput	
Melaleuca viridiflora	Niaouli	
Mentha piperita	Peppermint	
Myrtus communis	Myrtle	
Ocimum basilicum	Basil	
Origanum marjorana	Marjoram	Burgess and Finch (Cape Town, South Africa)
Origanum vulgare	Origanum	Prana Monde (Hainaut, Belgium)
Pimenta racemosa	Pimento	
Pinus sylvestris	Pine	
Piper nigrum	Black pepper	
Rosa damascena	Rose	
Rosmarinus officinalis	Rosemary	
Salvia officinalis	Sage	
Salvia sclarea	Clary sage	
Santalum austrocaledonicum	Sandalwood	Escentia Oils (Gauteng, South Africa)
Styrax benzoin	Benzoin	Scatters Oils (Gauteng, South Africa)
Syzygium caryophyllata	Clove	Prana Monde (Hainaut, Belgium)
Tagetes minuta	Tagetes	Escentia Oils (Gauteng, South Africa)
Thymus vulgaris	Thyme	Prana Monde (Hainaut, Belgium)
Zingiber officinale	Ginger	

Appropriate packaging and storage of essential oils is critical to maintaining the overall efficacy of the essential oils. Essential oils are sensitive to environmental conditions with temperature, light, and oxygen commonly recognized as having a crucial impact on essential oil integrity (Turek and Stintzing, 2013). In order to maintain essential oil stability, essential oils are stored in amber glass bottles with securely closing caps, to protect the essential oil from sunlight and other sources of UV radiation, and air. Therefore, the essential oils were stored at

4°C in sealed, airtight, amber vials until they were used for further analysis (Turek and Stintzing, 2013).

2.2. Micro-organism selection

The review of the literature provided in Article 1 (Leigh-de Rapper and van Vuuren, 2020) described the respiratory conditions cited by aromatherapists that could be managed and treated with essential oils. Based on the findings of this review, nine different respiratory pathogens were selected to determine the antimicrobial efficacy of the essential oils. A summary of the micro-organisms selected is provided in Table 2.2.

Table 2.2: Summary of micro-organisms selected for study

Micro-organism selected	Respiratory condition caused	Motivation for selection
Cryptococcus neoformans	Pulmonary cryptococcosis	Pulmonary cryptococcosis is a rare and severe pneumonia often isolated in immunocompromised patients, especially in patients with human immunodeficiency virus (HIV) infection (Kanjanapradit et al., 2017). South Africa has the largest HIV seropositive population in the world, with 7.8 million individuals living with HIV in 2022 (Be In The Know, 2022).
Haemophilus influenzae	Acute sinusitis, otitis media, bronchitis, pneumonia	A colonizer of the upper respiratory tract in up to 20% of infants, 50% of 5–6 year olds and 75% of most healthy adults (King, 2012). *Haemophilus influenzae* is a major cause of a variety of respiratory conditions including pneumonia (King, 2012).
Klebsiella pneumoniae	Pneumonia	Carried in the stool of 5 to 38% of individuals, while 1 to 6% is carried in the nasopharynx (Ashurst and Dawson, 2018). *Klebsiella pneumoniae* accounts for 11.8% of all hospital-acquired pneumonias and 3 to 15% of all community-acquired pneumonias in the world (Ashurst and Dawson, 2018).
Moraxella catarrhalis	Otitis media, bronchitis, pneumonia	An exclusively human pathogen and is responsible for 15-20% of all acute otitis media episodes in children (Murphy and Parameswaran, 2009).
*Mycobacterium tuberculosis**	Tuberculosis	Tuberculosis remains a leading respiratory infection responsible for killing approximately 1.7 million people in 2016 (WHO, 2017). The incidence rates of this infection remain high for South Africa with rates above 500/100 000 (WHO, 2017).

Micro-organism selected	Respiratory condition caused	Motivation for selection
Staphylococcus aureus	Pleural effusions, empyema, abscesses, pneumatoceles, necrotizing pneumonia	A commensal of the upper respiratory flora of up to 30% of normal individuals but is responsible for invasive opportunistic infection of the respiratory tract (Shukla et al., 2010). Lower respiratory tract infection due to *S. aureus* is less common than upper respiratory infections but are associated with high instances of mortality (Klevens et al., 2007).
Streptococcus agalactiae	Pneumonia	Recognized as a leading cause of postpartum infection and neonatal sepsis following pneumonia (Hanna and Noor, 2020).
Streptococcus pneumoniae	Acute sinusitis, otitis media, bronchitis, pneumonia	The most common bacterial cause of pneumonia in both adults and children (van Deursen et al., 2016).
Streptococcus pyogenes	Acute mastoiditis, epiglottitis, laryngitis, nasopharyngitis, rhinitis, tonsilitis, pneumonia	The most common bacterial cause of acute pharyngitis in both adults and children (Ashurst and Edgerley-Gibb, 2018).

*The avirulent, saprophytic surrogate *Mycobacterium smegmatis* was used for all susceptibility testing in place of *M. tuberculosis* due to the stringent and specific laboratory controls required to assess the highly virulent pathogen.

The antimicrobial activity of these essential oils is reported in **Article 2** (Leigh-de Rapper et al., 2021). The ethics waiver for investigating the antimicrobial activity is given in Appendix C.

2.3.Essential oil chemistry

Essential oils are complex mixtures of varying chemical compounds. The average essential oil could contain a few to hundreds of different individual chemical constituents (Ragno et al., 2021). These chemical compounds within essential oils often interact to create the synergistic or antagonistic relationships found between blends; however, some scientists believe that in certain instances, a single chemical entity may be responsible for the entire pharmacological action of a natural product (Tisserand and Young, 2013). Therefore, it is imperative when studying essential oils to determine the chemical composition of the essential oil studied as a means to 'fingerprint' the essential oil to its pharmacological effect. The chemical profiles of the essential oils selected for analysis is reported in **Article 2** (Leigh- de Rapper et al., 2021).

2.4. Essential oil structure-activity relationship

Chemometrics is the application of mathematical and statistical techniques used to retrieve significant information from complex datasets (Maree *et al*., 2014). Chemometrics is an invaluable tool for identifying patterns and determining structure-activity relationships within complex biological systems. Chemometrics employs the use of chemical data obtained from GC-MS analysis and compares that with laboratory data to determine compounds or combinations of compounds responsible for a perceived effect. The use of this software within essential oil research is used to identify single compounds or mixtures of compounds responsible for noteworthy antimicrobial activity. The structure-activity relationship of the essential oils cited for use in the respiratory tract is explored and reported in **Article 2** (Leigh-de Rapper et al., 2021).

Article 2 - Original article on essential oil antimicrobial and chemometric analysis

At the time of this thesis, this article had been published. This article is shown as it appears in publication.

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The use of chemometric modelling to determine chemical composition-antimicrobial activity relationships of essential oils used in respiratory tract infections

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ABSTRACT

The antimicrobial effects of essential oils are commonly cited within aromatherapeutic texts for use in respiratory tract infections. These essential oils are inhaled or applied to the skin to treat infections and manage symptoms associated with these conditions. A limited number of these essential oils have been scientifically studied to support these claims, specifically, against respiratory pathogens. This study reports on the minimum inhibitory concentration (MIC) of 49 commercial essential oils recommended for respiratory tract infections, and identifies putative biomarkers responsible for the determined antimicrobial effect following a biochemometric workflow. Essential oils were investigated against nine pathogens. Three essential oils, *Amyris balsamifera* (amyris), *Coriandrum sativum* (coriander) and *Santalum austrocaledonicum* (sandalwood) were identified as having greater activity (MIC value = 0.03–0.13 mg/ml) compared to the other essential oils investigated. The essential oil composition of all 49 oils were determined using Gas Chromatography coupled to Mass Spectroscopy (GC-MS) analysis and the GC-MS data analysed together with the antimicrobial data using chemometric tools. Eugenol was identified as the main biomarker responsible for antimicrobial activity in the majority of the essential oils. The ability of a chemometric model to accurately predict the active and inactive biomarkers of the investigated essential oils against pathogens of the respiratory tract was 80.33%.

1. Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) developed as a novel emerging respiratory illness in December 2019, having originated in Wuhan, China, from the Hunan seafood market, rapidly infecting more than 50 people [1,2]. A retrospective study from China found that 27 out of 28 patients with secondary bacterial respiratory infections died [3]. Previous to SARS-CoV-2, respiratory diseases were the leading cause of death and disability in the world with conditions such as pneumonia which were responsible for millions of deaths annually [4]. This continues in the era of SARS-CoV-2 with compounded, complicated secondary bacterial infections causing pneumonia and death.

Antibiotics have no efficacy in the treatment of viral respiratory infections, but are often prescribed as a result of patient expectations, with these agents leading to antimicrobial resistance [5]. Therefore, non-antibiotic alternative treatment options are needed to manage symptoms often associated with respiratory infections while treating patients with secondary infections caused by bacteria [6]. In order to address the effects of growing antimicrobial resistance, researchers have focused on investigations in alignment with natural products such as essential oils [7]. Commercially available aroma-therapeutic texts note extensively the use of essential oils for the treatment of respiratory infections [8–12]. Commonly prescribed essential oils for respiratory use include *Cinnamonum zeylanicum* (cinnamon), *Coriandrum sativum* (coriander), *Litsea cubeba* (may chang), *Melaleuca cajeput* (cajeput), *Origanum marjorana* (marjoram), *Szygium aromaticum* (clove) and *Zingiber officinale* (ginger) [8–12]. The antimicrobial susceptibility of essential oils has also been studied within the scientific literature extensively. The antimicrobial effects of the essential oils is primarily related to the specific

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essential oil chemistry. A study [13], indicated that the antimicrobial potential demonstrated by *Eucalyptus camaldulensis* was as a result of the high concentration of 1,8-cineole (>30%) identified in this essential oil. Additional research supports these findings with essential oils rich in chemical compounds such as carvacrol, cinnamaldhyde, eugenol and camphor identified as the bioactive antimicrobial molecules [14–17].

Essential oils are complex mixtures composed of different chemical compounds. The average essential oil could have anything between 18 chemical constituents such as in the case of *Citrus sinesis* [18] to 200 or more chemical constituents, as is found within the essential oil *Lavandula angustifolia* [19]. These chemical compounds within the crude essential oils often interact to create the synergistic or antagonistic relationships found between blends; however, some scientists believe that in certain instances, a single chemical entity may be responsible for the entire pharmacological action of a natural product [20]. Chemometrics is an invaluable tool for identifying patterns and determining structure-activity relationships within complex biological systems. Biochemometrics employs the use of chemical data obtained from sample chemistry and compares that with laboratory data of antimicrobial efficacy to determine compounds or combinations of compounds responsible for the perceived effect. The use of this approach within the field of antimicrobial research has potential to offer new opportunities in the study of essential oils [21–24]. The use of chemometric software has the potential to identify compounds responsible for antimicrobial activities and therefore guide the evidence-based use of essential oils for respiratory tract infections.

With this in mind, this study aimed to determine the *in vitro* antimicrobial activity of 49 commercial essential oils indicated in non-peer reviewed aroma-therapeutic texts for the management of respiratory infections and conditions [8–12], against nine pathogens of the respiratory tract to provide credibility to these recommendations. The selection of these oils was determined based on their in-text reference to use in respiratory tract infection management or the treatment of respiratory tract conditions and infections. Thereafter, the objective was to obtain chemical profiles of the essential oils using GC-MS analysis and to evaluate the antimicrobial findings hyphenated with the essential oil chemistry, using chemometric models.

2. Materials and methods

2.1. Essential oil selection

Forty-nine essential oils were included in this study whereby the selection was based on use for respiratory infections and the information was sourced from commercially available aroma-therapeutic texts [8–12]. These essential oils were obtained from international flavour and fragrance companies Robertet© (Grasse, France), Burgess and Finch (Cape Town, South Africa), PranaMonde (Belgium) and Escentia Oils (Gauteng, South Africa).

2.2. Gas chromatography coupled to mass spectrometry (GC-MS)/flame ionisation detector

The 49 selected essential oils were analysed using a GC-MS system (Agilent 6890N GC system coupled directly to a 5973 MS, Hanova, USA) equipped with a HP-Innowax polyethylene glycol column (60 m x 250 μm i.d. x 0.25 μm film thickness). A volume of 1 μl was injected (using a split ratio of 200:1) with an auto-sampler at 24.79 psi and an inlet temperature of 250 °C. The GC oven temperature was maintained at 60 °C for 10 min, then 220 °C at a rate of 4 °C/min for 10 min followed by a temperature of 240 °C at a rate of °C/min. The carrier gas, helium, was introduced at a constant flow of 1.2 ml/min. Spectra were obtained on electron impact at 70 eV, scanning from *m/z* 35 to 550. The percentage composition of the individual components were quantified by integration measurements using flame ionization detection (FID, 250 °C) and *n*-alkanes were used as reference points in the calculation of relative retention indices (RRI). Component identifications were made by comparing mass spectra from the total ion chromatogram, and retention indices using NIST and Mass Finder libraries [18].

2.3. Antimicrobial analysis

Microbial cultures were selected based on the relevance to respiratory infections and included the Gram-positive strains; *Staphylococcus aureus* (ATCC 25924), *Streptococcus agalactiae* (ATCC 55618), *Streptococcus pneumoniae* (ATCC 49619) and *Streptococcus pyogenes* (ATCC 12344). Selection was based on their pathogenesis to cause laryngitis, pneumonia and bronchitis [25]. The Gram-negative bacteria selected for this study included; *Haemophilus influenzae* (ATCC 19418), *Klebsiella pneumoniae* (ATCC 13883) and *Moraxella catarrhalis* (ATCC 23246). These strains were selected due to their pathogenesis to cause laryngitis and pneumonia, as well as their potential to cause nosocomial pneumonia in immunocompromised individuals, especially those with secondary infections resulting from new coronavirus, SARS-CoV-2 [26]. The condition tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is the most common lethal bacterial respiratory disease with over 10 million infections globally and 1.4 million deaths each year [4]. The pathogen *M. tuberculosis* requires stringent and specific laboratory controls due to the highly virulent nature of this pathogen. Studies by McGaw et al. [27] and Agrawal et al. [28] indicate the comparable drug sensitivity profiles of *Mycobacterium. tuberculosis* and *M. smegmatis* allowing for researchers to make use of more rapid growing, avirulent, saprophytic surrogate *Mycobacterium* species for antimicrobial susceptibility testing. The non-pathogenic *Mycobacterium* strain, *M. smegmatis* (ATCC 19420) was selected for this study as an indicator to determine the response that might occur among more pathogenic species of tuberculosis. The fungal strain, *Cryptococcus neoformans* (ATCC 14116), was selected due to its pathogenesis in immunocompromised hosts to cause fungal pneumonia and cryptococcosis [29,30].

All bacterial cultures, with the exception of the *Streptococcus* species, *H. influenzae* and *M. smegmatis*, were grown in Tryptone Soya broth (TSB; Oxoid), for 18 to 24 h at 37 °C. *Streptococcus agalactiae*, *S. pneumoniae*, *S. pyogenes* and *H. influenzae* were inoculated into *Haemophilus* test medium base with *Haemophilus* test medium supplement (HTM, ThermoFisher). *Streptococcus pneumoniae*, *S. pyogenes* and *H. influenzae* were incubated under anaerobic conditions at 5% CO_2 for 24 h at 37 °C, while *S. agalactiae* and the other test organisms were incubated under aerobic conditions for 24 h at 37 °C. *Mycobacterium smegmatis* was inoculated into Middlebrook broth with glycerol and ADC enrichment supplement (MB, ThermoFisher) and incubated under aerobic conditions for 48 h at 37 °C. *Cryptococcus neoformans* was grown in TSB and incubated at 37 °C for 48 h. All bacterial and fungal cultures, were streaked onto respective agar optimal for growth to confirm purity. A waiver for the use of these micro-organisms was granted by the University of the Witwatersrand Human Research Ethics Committee (Reference W-CJ-160720-2).

The broth microdilution method described by de Rapper et al. [18] was used to quantify the inhibitory activity of all the essential oils. The micro-titre plates were prepared by aseptically adding 100 μl of sterile broth (TSB for aerobic organisms, HTB for *H. influenzae* and the *Streptococcus* species or MB for *M. smegmatis*) into each of the wells of the plate. A stock concentration of each essential oil (32 mg/ml in acetone) was added to the first row at a volume of 100 μl. The essential oils were then serially diluted to concentrations from 8.00 to 0.06 mg/ml descending down each column of the micro-titre plate. The oils were tested in triplicate. A 100 μl volume of positive, negative and culture control was included for each micro-organism. The positive controls (Sigma–Aldrich) comprising of 0.01 mg/ml ciprofloxacin (for bacteria) or 0.1 mg/ml amphotericin B (for the yeast) were included to ensure microbial susceptibility. The negative control was 32 mg/ml water in acetone to exclude the attribution of antimicrobial effects due to the solvent. The culture controls ensured that the appropriate broths

supported growth. The micro-organisms for testing were prepared to an approximate inoculum concentration of 1 x 10^6 colony forming units per ml (CFU/ml). These were added to each well at a volume of 100 μl. Each micro-titre plate was sealed with sterile adhesive sealing film to ensure no loss of essential oil during testing due to the volatility of these samples. The micro-titre plates were incubated under optimal growth conditions relative to the test pathogen. After incubation, 40 μl of *p*-iodonitrotetrazolium violet solution (INT; 0.04% *w/v*; Sigma–Aldrich) was added to each well of the micro-titre plate and left at room temperature for 6–24 h, depending on the strain being tested. Viable micro-organisms interact with the INT solution to create a colour change from clear to pink. The lowest concentration with no colour change was considered the MIC value for that essential oil. For the essential oils, the minimum inhibitory concentrations (MICs) of ≤1.00 mg/ml are considered to have noteworthy antimicrobial activity [31].

2.4. Chemometric data analysis

The structure activity relationship (SAR) for each essential oil was determined using chemometric analysis [23]. Parallel to peak assignment and identification, the full scan GC-MS chromatograms were imported into MetAlignTM 4.1 software for pre-processing prior to chemometric analysis. Pre-processing involved baseline correction, denoising, noise elimination level of 2, mass window of 0.05, retention time window of 0.2, marker intensity threshold of 10, mass range was *m/z* 35–550 and the corresponding retention times were aligned across all samples [32]. The resulting aligned data of peak intensities was correlated to the antimicrobial activity for each essential oil and subjected to multivariate analysis using SIMCA-P+ 13.0 software (Umetrics, Umeå, Sweden). The unsupervised principal component analysis (PCA) was performed using a score plot and loading plot to provide an overview of all the samples with their constituents (observations) compared to their penetration depths (variables). This analysis allowed for the identification of groupings, clustering patterns, trends and outliers (observations that are different to the majority which is responsible for activity). Based on the initial intention to establish the structure activity relationship (SAR) of each essential oil, samples were pre-assigned to two different classes based on the MIC values for each pathogen. Distinct dummy Y-variables were assigned to samples which indicate the class of the sample. Samples with noteworthy antimicrobial activity (MIC values ≤ 1.00 mg/ml) were assigned to class 1 and samples with a less or poorer antimicrobial activity were assigned to class 2 (MIC values > 1.00 mg/ml). Following this, the data was analyzed separately for each pathogen using the supervised orthogonal projection to latent structures discriminant analysis (OPLS-DA). The OPLS model separates the systematic variation in the X-matrix (constituents of the samples that were determined via GC-MS) into one part which is linearly related to the Y-matrix and another that is unrelated to the Y-matrix. This analysis provides identification of the essential oil compounds responsible for perceived antimicrobial effect per investigated micro-organism. Therefore, nine discriminant analysis models (OPLS-DA) were constructed for each pathogen and the following parameters were used to evaluate each fitted model; the number of significant components, predictive variation in the X-matrix (R2Xp), orthogonal variation in the X-matrix (R2Xo) and cross-validated predictive ability of the model (Q2Ycum) [33]. Scatter score plots (tp1 vs. to1) displaying the distribution of active and poorly active essential oils was constructed for each pathogen. The corresponding S-plot for each model was explored to filter out putative retention time mass (RTM) pairs that could be correlated to the observed antimicrobial activity. The selected RTM pairs were used to identify the corresponding biomarkers (essential oil compounds) by comparing the MS spectra and RRI using various libraries [21].

2.5. Antimicrobial analysis of predicted chemometric biomarkers

Selected essential oil chemical constituents were purchased from Sigma-Aldrich based on their identification as putative biomarkers for good or poor antimicrobial activity, as per chemometric determination. The purity and percentage composition of the select chemical constituents were verified to the supplier's specifications by gas chromatography (minimum purity ≥95%). The antimicrobial effect of the select chemical constituents was determined as per MIC methodology previously outlined with the following modifications applied; chemical compounds were diluted to 32 mg/ml in acetone with the exception of viridiflorol diluted to 10 mg/ml in acetone and furanoeudesma-1,3-diene to 5.8 mg/ml in acetone. For the essential oil chemical constituents, the MICs of 0.06–0.10 μg/ml are clinically relevant while activities of 0.01 mg/ml or less are considered noteworthy in antimicrobial effect [34–36]. All MIC assays were undertaken at least in duplicate.

3. Results

3.1. Antimicrobial analysis

The antimicrobial efficacy of 49 commercial essential oils was investigated against nine pathogens associated to respiratory tract infections (Table 1). Noteworthy inhibition was observed for 31.9% of essential oils against all the pathogens tested. *Amyris balsamifera* (amyris), *C. sativum* and *C. zeylanicum* showed the broadest spectrum of activity (average MIC value of 0.68 mg/ml, 0.79 mg/ml and 0.97 mg/ml, respectively).

The yeast, *Cryptococcus neoformans*, was the most susceptible to the effects of the essential oils with 73.5% of essential oils investigated demonstrating noteworthy antimicrobial effects. Ten essential oils provided MIC results of less than 0.20 mg/ml with the most effective oils determined as *C. sativum* (MIC of 0.03 mg/ml), *Syzygium caryophyllata* (clove; with an MIC of 0.06 mg/ml), *Helichrysum italicum* (imortelle), *Hyssopus officinalis* var. *officinalis* (hyssop), *Melaleuca cajuputii* (cajeput), *Pinus sylvestris* (pine), *Salvia officinalis* (sage) and *Salvia sclarea* (clary sage) (all with MIC values of 0. 13 mg/ml), *Santalum austrocaledonicum* (sandalwood) and *Thymus vulgaris* (thyme) (with MIC values of 0. 19 mg/ml).

Streptococcus pyogenes was the bacterial pathogen most susceptible to the effects of the essential oils with 22 of the 49 essential oils investigated demonstrating noteworthy antimicrobial activity. The essential oil most effective against this pathogen was *S. austrocaledonicum* with an MIC value as low as 0.06 mg/ml.

3.2. Essential oil chemistry

The major compounds identified in each of the essential oils are given in Table 2. Although the full chemical profile was established for each essential oil and incorporated into the chemometric analysis, a summarized chemical profile with major compounds (>10%) was provided in Table 2 for the sake of brevity. The essential oil chemotypes identified are consistent with that reported elsewhere in the literature.

3.3. Chemometric analysis

Principal component analysis was applied to the essential oil GC-MS data. The principal component analyses (PCA) scores scatter plot reveals the possible presence of outliers, groups, similarities and other patterns in the data and is essentially a map of the observations [76]. Outliers are observations that are situated outside the ellipses and appear to break the pattern or grouping shown by the majority of the data. These data points are due to random variation in measurements or may be of more scientific importance [77]. Moderate outliers are considered to be those that stay near the margin of the ellipses, while those further away are considered more severe [77]. Moderate outliers with distinct chemical profiles for *Lavandula angustifolia* (lavender), *Lavandula burnati reydovan* (lavandin), *Citrus bergamia* (bergamot) and *Daucus carota* (carrot seed) essential oils were determined (Fig. 1).

Table 1
The antimicrobial activity of 49 essential oils against pathogens of the respiratory tract

Essential oils		Minimum inhibitory concentration (Std. deviation) in mg/ml (n=3)								
		Gram-positive bacteria					Gram-negative bacteria			Yeast
Scientific name	Common name	*S. au** ATCC 25924	*S. ag* ATCC 55618	*S. pn* ATCC 49619	*S. py* ATCC 12344	*M. sm* ATCC 19420	*H. in* ATCC 19418	*K. pn* ATCC 13883	*M. ca* ATCC 23246	*C. ne* ATCC 14116
Abies balsamea [c]	Fir	2.00 (± 0.00)	2.00 (± 0.00)	**1.00 (± 0.00)**	3.00 (± 1.00)	2.00 (± 0.00)	1.50 (± 0.50)	**1.00 (± 0.00)**	2.00 (± 0.00)	4.00 (± 0.00)
Amyris balsamifera [c]	Amyris	**0.50 (± 0.00)**	**0.50 (± 0.00)**	**0.13 (± 0.00)**	**0.38 (± 0.25)**	**1.00 (± 0.00)**	**0.13 (± 0.00)**	2.00 (± 0.00)	**1.00 (± 0.00)**	**0.50 (± 0.00)**
Boswellia carteri [c]	Frankincense	8.00 (± 0.00)	2.00 (± 0.00)	**1.00 (± 0.00)**	2.00 (± 0.00)	3.00 (± 1.00)	**0.50 (± 0.00)**	2.00 (± 0.00)	2.00 (± 0.00)	**1.00 (± 0.00)**
Canarium luzonicum [c]	Elemi	2.00 (± 0.00)	2.00 (± 0.00)	2.00 (± 0.00)	1.50 (± 0.50)	4.00 (± 0.00)	3.00 (± 1.00)	2.00 (± 0.00)	2.00 (± 0.00)	**0.50 (± 0.00)**
Carum carvi [c]	Caraway	2.00 (± 0.00)	2.00 (± 0.00)	2.00 (± 0.00)	2.00 (± 0.00)	4.00 (± 0.00)	1.50 (± 0.50)	2.00 (± 0.00)	2.00 (± 0.00)	**0.50 (± 0.00)**
Cinnamomum camphora [c]	Camphor	**1.00 (± 0.00)**	2.00 (± 0.00)	3.00 (± 1.00)	2.00 (± 0.00)	8.00 (± 0.00)	**1.00 (± 0.00)**	**1.00 (± 0.00)**	2.00 (± 0.00)	4.00 (± 0.00)
Cinnamomum zeylanicum [c]	Cinnamon	**1.00 (± 0.00)**	**0.50 (± 0.00)**	2.00 (± 0.00)	**0.25 (± 0.00)**	2.00 (± 0.00)	**0.75 (± 0.25)**	**1.00 (± 0.00)**	**0.50 (± 0.00)**	**0.75 (± 0.25)**
Citrus aurantifolia [d]	Lime	4.00 (± 0.00)	2.00 (± 0.00)	2.00 (± 0.00)	2.00 (± 0.00)	4.00 (± 0.00)	8.00 (± 0.00)	2.00 (± 0.00)	2.00 (± 0.00)	**0.75 (± 0.25)**
Citrus bergamia [c]	Bergamot	8.00 (± 0.00)	2.00 (± 0.00)	2.00 (± 0.00)	3.00 (± 1.00)	4.00 (± 0.00)	3.00 (± 1.00)	3.00 (± 1.00)	8.00 (± 0.00)	**1.00 (± 0.00)**
Citrus limon [c]	Lemon	8.00 (± 0.00)	2.00 (± 0.00)	2.00 (± 0.00)	2.00 (± 0.00)	4.00 (± 0.00)	8.00 (± 0.00)	3.00 (± 1.00)	**1.00 (± 0.00)**	1.50 (± 0.50)
Citrus sinensis [c]	Orange	8.00 (± 0.00)	2.00 (± 0.00)	2.00 (± 0.00)	**1.00 (± 0.00)**	4.00 (± 0.00)	8.00 (± 0.00)	2.00 (± 0.00)	3.00 (± 1.00)	**1.00 (± 0.00)**
Commiphora molmol [c]	Myrrh	4.00 (± 0.00)	2.00 (± 0.00)	**1.00 (± 0.00)**	**0.19 (± 0.06)**	**1.00 (± 0.00)**	**0.25 (± 0.00)**	8.00 (± 0.00)	2.00 (± 0.00)	**0.50 (± 0.00)**
Coriandrum sativum [c]	Coriander	**0.50 (± 0.00)**	**1.00 (± 0.00)**	**1.00 (± 0.00)**	**0.25 (± 0.00)**	2.00 (± 0.00)	**0.38 (± 0.13)**	**0.50 (± 0.00)**	1.50 (± 0.50)	**0.03 (± 0.00)**
Cupressus sempervirens var. *stricta* [c]	Cypress	3.00 (± 1.00)	2.00 (± 0.00)	8.00 (± 0.00)	3.00 (± 1.00)	4.00 (± 0.00)	3.00 (± 1.00)	2.00 (± 0.00)	8.00 (± 0.00)	**0.50 (± 0.00)**
Cymbopogon citratus [c]	Lemongrass	**1.00 (± 0.00)**	2.00 (± 0.00)	**1.00 (± 0.00)**	**0.38 (± 0.13)**	2.00 (± 0.00)	1.50 (± 0.50)	8.00 (± 0.00)	2.00 (± 0.00)	8.00 (± 0.00)
Daucus carota [c]	Carrot seed	2.00 (± 0.00)	2.00 (± 0.00)	**1.00 (± 0.00)**	**1.00 (± 0.00)**	4.00 (± 0.00)	**0.25 (± 0.00)**	2.00 (± 0.00)	2.00 (± 0.00)	**0.38 (± 0.13)**
Elettaria cardamonum [c]	Cardamom	**1.00 (± 0.00)**	2.00 (± 0.00)	2.00 (± 0.00)	2.00 (± 0.00)	8.00 (± 0.00)	3.00 (± 1.00)	**1.00 (± 0.00)**	2.00 (± 0.00)	1.50 (± 0.50)
Eucalyptus globulus [c]	Eucalyptus	4.00 (± 0.00)	2.00 (± 0.00)	1.50 (± 0.50)	2.00 (± 0.00)	4.00 (± 0.00)	3.00 (± 1.00)	2.00 (± 0.00)	2.00 (± 0.00)	**1.00 (± 0.00)**
Foeniculum vulgare [c]	Fennel	**1.00 (± 0.00)**	2.00 (± 0.00)	**1.00 (± 0.00)**	3.00 (± 1.00)	3.00 (± 1.00)	3.00 (± 1.00)	**1.00 (± 0.00)**	2.00 (± 0.00)	2.00 (± 0.00)
Helichrysum italicum [c]	Imortelle	4.00 (± 0.00)	2.00 (± 0.00)	2.00 (± 0.00)	1.50 (± 0.50)	2.00 (± 0.00)	4.00 (± 0.00)	6.00 (± 2.00)	1.50 (± 0.50)	**0.13 (± 0.00)**
Hyssopus officinalis var. *officinalis* [c]	Hyssop	2.00 (± 0.00)	2.00 (± 0.00)	2.00 (± 0.00)	**1.00 (± 0.00)**	4.00 (± 0.00)	8.00 (± 0.00)	2.00 (± 0.00)	2.00 (± 0.00)	**0.13 (± 0.00)**
Illicium verum [c]	Star-anise	**1.00 (± 0.00)**	2.00 (± 0.00)	2.00 (± 0.00)	**1.00 (± 0.00)**	0.75 (± 0.25)	1.50 (± 0.50)	**1.00 (± 0.00)**	2.00 (± 0.00)	4.00 (± 0.00)
Juniperus virginiana [c]	Cedarwood	**0.50 (± 0.00)**	**1.00 (± 0.00)**	2.00 (± 0.00)	**0.19 (± 0.06)**	2.00 (± 0.00)	**0.50 (± 0.00)**	2.00 (± 0.00)	**1.00 (± 0.00)**	**0.50 (± 0.00)**
Laurus nobilis [c]	Bay	**1.00 (± 0.00)**	2.00 (± 0.00)	4.00 (± 0.00)	3.00 (± 1.00)	4.00 (± 0.00)	4.00 (± 0.00)	1.50 (± 0.50)	8.00 (± 0.00)	4.00 (± 0.00)
Lavandula angustifolia [a]	Lavender	**1.00 (± 0.00)**	2.00 (± 0.00)	**1.00 (± 0.00)**	4.00 (± 0.00)	2.00 (± 0.00)	3.00 (± 1.00)	1.50 (± 0.50)	2.00 (± 0.00)	**1.00 (± 0.00)**
Lavandula burnati [b]	Lavandin	1.50 (± 0.50)	2.00 (± 0.00)	**1.00 (± 0.00)**	**1.00 (± 0.00)**	2.00 (± 0.00)	2.00 (± 0.00)	**1.00 (± 0.00)**	2.00 (± 0.00)	1.50 (± 0.50)
Lavandula spica [c]	Lavender spike	**1.00 (± 0.00)**	2.00 (± 0.00)	1.50 (± 0.50)	3.00 (± 1.00)	4.00 (± 0.00)	4.00 (± 0.00)	1.50 (± 0.50)	**1.00 (± 0.00)**	1.50 (± 0.50)
Melaleuca alternifolia [b]	Tea tree	4.00 (± 0.00)	1.50 (± 0.50)	2.00 (± 0.00)	1.50 (± 0.50)	4.00 (± 0.00)	1.50 (± 0.50)	4.00 (± 0.00)	2.00 (± 0.00)	**0.75 (± 0.25)**
Melaleuca cajuputii [c]	Cajeput	4.00(± 0.00)	2.00 (± 0.00)	3.00 (± 1.00)	4.00 (± 0.00)	4.00 (± 0.00)	**1.00 (± 0.00)**	2.00 (± 0.00)	2.00 (± 0.00)	**0.13 (± 0.00)**
Melaleuca viridiflora [c]	Niaouli	4.00 (± 0.00)	2.00 (± 0.00)	**1.00 (± 0.00)**	**1.00 (± 0.00)**	4.00 (± 0.00)	8.00 (± 0.00)	2.00 (± 0.00)	2.00 (± 0.00)	**0.25 (± 0.00)**
Mentha piperita [c]	Peppermint	**1.00 (± 0.00)**	2.00 (± 0.00)	4.00 (± 0.00)	1.50 (± 0.50)	4.00 (± 0.00)	2.00 (± 0.00)	**1.00 (± 0.00)**	2.00 (± 0.00)	1.50 (± 0.50)
Myrtus communis [c]	Myrtle	**4.00 (± 0.00)**	2.00 (± 0.00)	2.00 (± 0.00)	8.00 (± 0.00)	6.00 (± 2.00)	6.00 (± 2.00)	**2.00 (± 0.00)**	2.00 (± 0.00)	2.00 (± 0.00)
Ocimum basilicum [c]	Basil	**0.50 (± 0.00)**	**1.00 (± 0.00)**	2.00 (± 0.00)	1.50 (± 0.50)	4.00 (± 0.00)	6.00 (± 2.00)	**1.00 (± 0.00)**	**1.00 (± 0.00)**	**0.25 (± 0.00)**
Origanum marjorana [b]	Marjoram	8.00 (± 0.00)	2.00 (± 0.00)	2.00 (± 0.00)	**1.00 (± 0.00)**	3.00 (± 1.00)	3.00 (± 1.00)	2.00 (± 0.00)	2.00 (± 0.00)	2.00 (± 0.00)
Origanum vulgare [c]	Origanum									

(*continued on next page*)

Table 1 (*continued*)

Essential oils		Minimum inhibitory concentration (Std. deviation) in mg/ml (n=3)								
		Gram-positive bacteria					Gram-negative bacteria			Yeast
Scientific name	Common name	*S. au** ATCC 25924	*S. ag* ATCC 55618	*S. pn* ATCC 49619	*S. py* ATCC 12344	*M. sm* ATCC 19420	*H. in* ATCC 19418	*K. pn* ATCC 13883	*M. ca* ATCC 23246	*C. ne* ATCC 14116
Pimenta racemose [c]	Pimento	**1.00 (± 0.00)**	2.00 (± 0.00)	4.00 (± 0.00)	**1.00 (± 0.00)**	4.00 (± 0.00)	3.00 (± 1.00)	2.00 (± 0.00)	2.00 (± 0.00)	**1.00 (± 0.00)**
Pinus sylvestris [c]	Pine	**0.50 (± 0.00)**	2.00 (± 0.00)	**1.00 (± 0.00)**	**0.50 (± 0.00)**	2.00 (± 0.00)	1.50 (± 0.50)	**0.50 (± 0.00)**	**1.00 (± 0.00)**	**0.25 (± 0.00)**
Piper nigrum [c]	Black pepper	2.00 (± 0.00)	2.00 (± 0.00)	**1.00 (± 0.00)**	1.50 (± 0.50)	8.00 (± 0.00)	8.00 (± 0.00)	2.00 (± 0.00)	2.00 (± 0.00)	**0.13 (± 0.00)**
Rosa damascena [c]	Rose	8.00 (± 0.00)	2.00 (± 0.00)	3.00 (± 1.00)	3.00 (± 1.00)	4.00 (± 0.00)	2.00 (± 0.00)	2.00 (± 0.00)	2.00 (± 0.00)	**0.25 (± 0.00)**
Rosmarinus officinalis [c]	Rosemary	2.00 (± 0.00)	2.00 (± 0.00)	2.00 (± 0.00)	**0.13 (± 0.00)**	2.00 (± 0.00)	**1.00 (± 0.00)**	2.00 (± 0.00)	2.00 (± 0.00)	**0.50 (± 0.00)**
Salvia officinalis [d]	Sage	4.00 (± 0.00)	2.00 (± 0.00)	2.00 (± 0.00)	**1.00 (± 0.00)**	4.00 (± 0.00)	8.00 (± 0.00)	2.00 (± 0.00)	2.00 (± 0.00)	**0.25 (± 0.00)**
Salvia sclarea [c]	Clary sage	1.50 (± 0.50)	2.00 (± 0.00)	2.00 (± 0.00)	**1.00 (± 0.00)**	4.00 (± 0.00)	3.00 (± 1.00)	**1.00 (± 0.00)**	2.00 (± 0.00)	**0.13 (± 0.00)**
Santalum austrocaledonicum [c]	Sandalwood	8.00 (± 0.00)	2.00 (± 0.00)	8.00 (± 0.00)	3.00 (± 1.00)	4.00 (± 0.00)	3.00 (± 1.00)	8.00 (± 0.00)	8.00 (± 0.00)	**0.13 (± 0.00)**
Styrax benzoin [d]	Benzoin	**0.75 (± 0.25)**	**0.25 (± 0.00)**	2.00 (± 0.00)	**0.06 (± 0.00)**	**0.75 (± 0.25)**	**0.13 (± 0.00)**	8.00 (± 0.00)	**0.25 (± 0.00)**	**0.19 (± 0.06)**
Syzygium caryophyllata [c]	Clove	4.00 (± 0.00)	2.00 (± 0.00)	4.00 (± 0.00)	1.50 (± 0.50)	4.00 (± 0.00)	2.00 (± 0.00)	2.00 (± 0.00)	2.00 (± 0.00)	**0.38 (± 0.13)**
Tagetes minuta [d]	Tagetes	2.00 (± 0.00)	**1.00 (± 0.00)**	**1.00 (± 0.00)**	2.00 (± 0.00)	2.00 (± 0.00)	**0.25 (± 0.00)**	2.00 (± 0.00)	**1.00 (± 0.00)**	**0.06 (± 0.00)**
Thymus vulgaris [c]	Thyme	**1.00 (± 0.00)**	2.00 (± 0.00)	4.00 (± 0.00)	**0.50 (± 0.00)**	2.00 (± 0.00)	3.00 (± 1.00)	**1.00 (± 0.00)**	2.00 (± 0.00)	**1.00 (± 0.00)**
Zingiber officinale [c]	Ginger	4.00 (± 0.00)	**1.00 (± 0.00)**	4.00 (± 0.00)	**0.50 (± 0.00)**	4.00 (± 0.00)	**0.50 (± 0.00)**	2.00 (± 0.00)	**0.19 (± 0.06)**	
Ciprofloxacin µg/ml		**1.00 (± 0.00)**	**0.50 (± 0.00)**	**2.00 (± 0.00)**	**1.00 (± 0.00)**	**2.00 (± 0.00)**	**3.00 (± 1.00)**	**4.00 (± 0.00)**	**1.00 (± 0.00)**	**0.50 (± 0.00)**
Amphotericin B µg/ml		**0.50 x 10^{-3}**	**0.50 x 10^{-3}**	**0.50 x 10^{-3}**	**0.50 x 10^{-3}**	**0.50 x 10^{-3}**	**0.50 x 10^{-3}**	**0.25 x 10^{-3}**	**1.00 x 10^{-3}**	**n.a.**
		n.a.	**n.a.**	**n.a.**	**n.a.**	**n.a.**	**n.a.**	**n.a.**	**n.a.**	**0.50 x 10^{-3}**

* ***S. au- Staphylococcus aureus, S. ag – Streptococcus agalactiae, S. pn – Streptococcus pneumoniae, S. py – Streptococcus pyogenes, H. in – Haemophilus influenzae, K. pn – Klebsiella pneumoniae, M. ca – Moraxella*** *catarrhalis, M. sm – Mycobacterium smegmatis, C. ne – Cryptococcus neoformans.*. **Bold –** noteworthy antimicrobial activity [23]. These essential oils were obtained from international flavour and fragrance companies Robertet© (Grasse, France)[a], Burgess and Finch (Cape Town, South Africa)[b], PranaMonde (Belgium)[c] and Escentia Oils (Gauteng, South Africa)[d].

The distribution of the essential oils on the scores scatter plot is a visual representation of essential oil chemistry identified from chromatographic data. Essential oils grouped together are chemically more similar than those grouped further away. When comparing the outlier essential oils with those of the grouped essential oils, it was observed that the outlier oils had chemical profiles different from many of the essential oils studied. The essential oils distributed away from the ellipses as outliers had linalyl acetate (*L. angustifolia* (35,6%) and *L. burnati* (17,8%)) and linalyl formate (*C. bergamia* (33,0%)), while *D. carota* comprises of carotol (42,1%) in majority. As these oils are obtained from diverse botanical species from different plant families and genera the diversity in oil composition as visually reflected in Fig. 1 is to be expected.

The chemical composition-antimicrobial activity relationships of the essential oils were determined through application of orthogonal projections to latent structures discriminant analysis (OPLS-DA). Correlation of essential oil chemistry and antimicrobial activity is only possible where two or more distinct classes can be defined in a dataset [23]. The OPLS-DA models for all pathogens were developed and presented in Table 3.

For illustrative purposes, two OPLS-DA models (*M. catarrhalis* and *C. neoformans*) are graphically presented and explained in detail in the results. These models were selected for graphical representation as *M. catarrhalis* which showed the strongest model fit with the most resistant antimicrobial activity while *C. neoformans* showed the weakest model fit with the most susceptible antimicrobial activity. For the pathogens, *M. catarrhalis* and *C. neoformans*, a scores scatter plot of the OPLS-DA data (Fig. 2) was generated with each model fitted with one predictive and one orthogonal component (A: 1 + 1). The total variance modelled for the scores scatter plot generated for *M. catarrhalis* was 6.7% (R2Xcum = 0.067), while the scores scatter plot for *C. neoformans* showed a cumulative change of 5.8% (R2Xcum = 0.058).

From the scores scatter plot generated, clear separation between the active (green/left) and inactive oils (blue/right) were observed along the predictive component (x-axis) with a 3.5% chemical variation (R2Xp = 0.0356) being attributed to this classification for the pathogen *M. catarrhalis*. In the majority of the essential oils tested, most were inactive against *M. catarrhalis* with 22% of the essential oils classified as active against this pathogen. The same clear separation between the active (green/left) and inactive oils (blue/right) was observed for the scores scatter plot generated for the pathogen *C. neoformans*, however, only a 2.2% chemical variation (R2Xp = 0.0219) was attributed to the classification of active from inactive oils. The essential oils showed greater antimicrobial efficacy against this pathogen with 73% of the essential oils classified as active. For all nine fitted OPLS-DA models, two principal components were used. The variation models for each pathogen is very low with all models showing predictive variation of less or equal to 3% (R^2Xp) and a cumulative variation of less than 6% (R^2Xcum).

To identify essential oil compounds that have a significant contribution to the antimicrobial activity, an S-plot of the variables was plotted (Fig. 3). In the *S*-plot, the extreme ends of the plot identify variables that are statistically significant and highly reliable discriminators of the two classes [78]. The highlighted variables on the left-hand

Table 2
Identified major essential oil chemical constituents

Essential oil		Chemical constituents				Reference
Scientific name	Common name	Identified in this study		Identified in literature		
		29.8%	β-Pinene	29.9%	β-Pinene	
		17.5%	δ-3-Carene	19.6%	δ-3-Carene	
Abies balsamea [c]	Fir	11.6%	Bornyl acetate	14.6%	α-Pinene	[37]
		10.4%	β-Eudesmol	23.6%	7-epi-D-Eudesmol	
		11.8%	α-Panasinsen	12.3%	λ-Eudesmol	
Amyris balsamifera [c]	Amyris			14.0%	Agarospirol	[38]
		47.9%	α-Pinene	37.3%	α-Pinene	
Boswellia carterii [c]	Frankincense	12.8%	Limonene			[39]
		47.5%	Limonene	56.0%	Limonene	
Canarium luzonicum [c]	Elemi	18.4%	Elemol			[40]
		64.1%	Carvone	78.9%	Carvone	
Carum carvi [c]	Caraway	34.0%	Limonene			[41]
		41.1%	1,8-Cineole	41.1%	1,8-Cineole	
Cinnamomum camphora [c]	Camphor	27.5%	Limonene	27.5%	Limonene	[23]
Cinnamomum zeylanicum [c]	Cinnamon	78.4%	Eugenol	80.0%	Eugenol	[18]
Citrus aurantifolia [c]	Lime	16.4%	α-Terpineol	12.7%	α-Terpineol	[42]
		11.2%	Terpinolene	40.4%	Limonene	
		39.1%	Limonene	33.7%	Limonene	
Citrus bergamia [c]	Bergamot	33.0%	Linalyl formate			[43]
		74.9%	Limonene	61.7%	Limonene	
Citrus limon [c]	Lemon			10.2%	β-Pinene	[44]
Citrus sinensis [c]	Orange	93.5%	Limonene	90.7%	Limonene	[45]
Commiphora molmol [c]	Myrrh	52.9%	Furanoeusdema-1,3-diene	57.7%	Furanoeusdema-1,3-diene	[46]
		15.8%	Lindestrene	16.3%	Lindestrene	
Coriandrum sativum [c]	Coriander	72.4%	Linalool	72.3%	Linalool	[41]
		50.9%	α-Pinene	22.9%	α-Pinene	
Cupressus sempervirens var. *stricta* [c]	Cypress	22.5%	δ-3-Carene			[47]
		42.7%	Geranial	40.7%	Geranial	
		28.9%	Neral	30.6%	Neral	
Cymbopogon citratus [c]	Lemongrass	11.7%	Limonene			[48]
Daucus carota [c]	Carrot seed	42.1%	Carotol	48.0%	Carotol	[49]
		56.1%	Terpinyl acetate	46.1%	Terpinyl acetate	
Elettaria cardamonum [c]	Cardamom	24.1%	1,8-Cineole			[50]
		63.6%	1,8-Cineole	63.8%	1,8-Cineole	
Eucalyptus globulus [c]	Eucalyptus	10.5%	α-Terpineol			[51,52]
Ferula galbaniflua [c]	Galbanum	54.3%	β-Pinene	58.8%	β-Pinene	[53]
Foeniculum vulgare [c]	Fennel	82.0%	*(E)*-Anethole	80.7%	*(E)*-Anethole	[54]
		12.4%	β-Caryophyllene	18.6%	β-Caryophyllene	
		19.4%	1,8-Cineole			
Helichrysum italicum [c]	Immortelle	15.0%	α-Humulene			[55]
		47.9%	Isopinocamphone	48.7%	Isopinocamphone	
		15.1%	Pinocamphone	15.5%	Pinocamphone	
Hyssopus officinalis var. *officinalis* [c]	Hyssop	10.1%	β-Pinene			[18]
Illicium verum [c]	Star-anise	79.8%	*(E)*-Anethole	75.7%	*(E)*-Anethole	[56]
		22.4%	α-Pinene	20.5%	α-Pinene	
		15.4%	Benzyl benzoate	13.7%	Benzyl benzoate	
Juniperus virginiana [c]	Cederwood	11.5%	Myrcene			[18]
		54.4%	Eugenol	57.2%	Eugenol	
		18.5%	Myrcene	14.3%	Myrcene	
Laurus nobilis [c]	Bay	11.5%	Chavicol	12.7%	Chavicol	[18]
		32.8%	Linalool	40.3%	Linalool	
		35.6%	Linalyl acetate			
Lavandula angustifolia [a]	Lavender	10.2%	β-Caryophyllene			[57,58]
		60.3%	Linalool	54.0%	Linalool	
Lavandula burnati [b]	Lavandin	17.8%	Linalyl acetate			[58]
		43.1%	Linalool	43.8%	Linalool	
		26.0%	1,8-Cineole	31.8%	1,8-Cineole	
Lavandula spica [c]	Lavender spike	10.9%	Camphor	12.0%	Camphor	[59]
		44.6%	Terpinen-4-ol	23.1%	Terpinen-4-ol	
Melaleuca alternifolia [b]	Tea tree	16.6%	γ-Terpinene			[60]
		56.7%	1,8-Cineole	67.6%	1,8-Cineole	
		14.2%	Limonene			
Melaleuca cajuputii [c]	Cajeput	10.6%	Benzyl alcohol			[51]
		49.8%	Linalyl acetate	49.8%	Linalyl acetate	
Melaleuca viridiflora [c]	Niaouli	30.1%	Linalool	30.1%	Linalool	[23]
		47.0%	Menthol	47.5%	Menthol	
Mentha piperita [c]	Peppermint	18.8%	Menthone	27.9%	Menthone	[61,62]
		27.4%	1,8-Cineole	52.5%	1,8-Cineole	
		22.1%	Myrtenyl acetate	26.3%	Myrtenyl acetate	
		16.7%	α-Pinene	9.5%	α-Pinene	
Myrtus communis [c]	Myrtle	10.0%	Limonene			[63]
		74.2%	Estragole	61.0%	Estragole	
Ocimum basilicum [c]	Basil	18.4%	Linalool			[64]

(*continued on next page*)

Table 2 (*continued*)

Essential oil		Chemical constituents				Reference
Scientific name	Common name	Identified in this study		Identified in literature		
		51.9%	1,8-Cineole	46.0%	1,8-Cineole	
Origanum marjorana [b]	Marjoram	22.4%	Linalyl formate	26.1%	Linalool	[18]
Origanum vulgare [c]	Origanum	67.9%	Carvacrol	65.9%	Carvacrol	[65]
Pimenta racemosa [c]	Pimento	56.1%	Eugenol	74.7%	Eugenol	[66]
		19.3%	Myrcene			
Pinus sylvestris [c]	Pine	34.8%	Bornyl acetate	25.0%	Bornyl acetate	[18,67]
		22.3%	Camphene	11.8%	Camphene	
		11.1%	α-Pinene	11.0%	α-Pinene	
		28.0%	β-Caryophyllene	29.5%	β-Caryophyllene	
		18.1%	Limonene	18.7%	Limonene	
Piper nigrum [c]	Black pepper	10.7%	β-Pinene	19.2%	δ-3-Carene	[68]
		29.9%	Citronellol	42.0%	Citronellol	
		20.8%	Geraniol	28.5%	(*E*)-Geraniol	
		22.4%	Phenelyl alcohol	19.9%	Nonadecane	
Rosa damascena [c]	Rose	10.2%	Nerol			[69]
		19.1%	Camphor	19.6%	Camphor	
		14.7%	α-Pinene	11.7%	1,8-Cineole	
		12.3%	Verbenone	11.5%	Verbenone	
Rosmarinus officinalis [c]	Rosemary	12.2%	Bornyl acetate	10.6%	Borneol	[70]
		24.9%	α-Thujone	28.2%	α-Thujone	
Salvia officinalis [d]	Sage	19.8%	Camphor			[71]
		58.5%	Linalyl acetate	40.6%	Linalyl acetate	
Salvia sclarea [c]	Clary sage	23.5%	Linalool	26.0%	Linalool	[72]
		52.7%	α-Santalol	32.1%	α-Santalol	
Santalum austrocaledonicum [c]	Sandalwood	15.7%	β-Santalol			[18]
Styrax benzoin [d]	Benzoin	72.2%	Dimethyl phthalate	72.0%	Dimethyl phthalate	[23]
		81.9%	Eugenol	66.9%	Eugenol	
Syzygium caryophyllata [c]	Clove	13.1%	Isoeugenol			[73]
		47.8%	(*E*)-β-Ocimene	52.0%	(*E*)-β-Ocimene	
Tagetes minuta [d]	Tagetes	10.9%	(*E*)-Tagetone			[74]
		41.0%	p-Cymene	35.7%	p-Cymene	
Thymus vulgaris [c]	Thyme	18.9%	Thymol	33.2%	Thymol	[75]
		37.6%	α-Curcumeme	37.6%	α-Curcumeme	
Zingiber officinale [c]	Ginger	14.4%	Himachalene	14.4%	Himachalene	[23]

These essential oils were obtained from international flavour and fragrance companies Robertet© (Grasse, France)[a], Burgess and Finch (Cape Town, South Africa)[b], PranaMonde (Belgium)[c] and Escentia Oils (Gauteng, South Africa)[d].

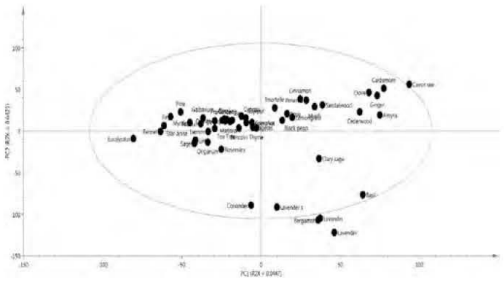


Fig. 1. Principal component scores scatter plot for 49 commercial essential oils used for the treatment and management of respiratory tract infections (common names used for ease of visualization).

bottom wing of the S-shape (green square) are indicative of essential oil biomarkers correlated to active essential oils while variables on the top right-hand wing of the S-shape (blue square) are associated to putative biomarkers of the inactive class.

A list of the active (MIC values ≤ 1.00 mg/ml) and inactive (MIC values > 1.00 mg/ml) variables were determined using the biomarker retention time/mass pairs (from GC-MS data) to identify the essential oil chemical compound via NIST library (Tables 4 and 5).

Eugenol was the identified putative biomarker responsible for the noteworthy antimicrobial activity against a wide variety of test organisms including *S. pneumoniae*, *S. pyogenes*, *K. pneumoniae* and *M. catarrhalis*. Other repetitive biomarkers included thymol

Table 3
OPLS-DA model statistics against all nine pathogens

Pathogens	A[a]	R^2Xp[b]	R^2X (cum)[c]	R^2Y (cum)[d]	Q^2(cum)[e]
Staphylococcus aureus (ATCC 25924)	2 (1 + 1)	0.035	0.062	0.931	0.061
Streptococcus agalactiae (ATCC 55618)	2 (1 + 1)	0.030	0.066	0.922	- 0.054
Streptococcus pneumoniae (ATCC 49619)	2 (1 + 1)	0.028	0.063	0.933	0.139
Streptococcus pyogenes (ATCC 12344)	2 (1 + 1)	0.030	0.067	0.941	0.100
Mycobacterium smegmatis (ATCC 23246)	2 (1 + 1)	0.028	0.061	0.965	- 0.012
Haemophilus influenzae (ATCC 19420)	2 (1 + 1)	0.029	0.063	0.920	0.017
Klebsiella pneumoniae (ATCC 19418)	2 (1 + 1)	0.026	0.057	0.913	- 0.158
Moraxella catarrhalis (ATCC 13883)	2 (1 + 1)	0.031	0.067	0.929	0.279
Cryptococcus neoformans (ATCC 14116)	2 (1 + 1)	0.022	0.058	0.904	- 0.097

[a] Number of principal components (Predictive + Orthogonal).
[b] Predictive variation in X that is explained by the model.
[c] The sum of variation in X that is explained by the model.
[d] The sum of variation in Y that is explained by the model.
[e] Ability to predict future outcomes.

(*S. agalactiae, H. influenzae*), *cis*-β-ocimene (*S. aureus, K. pneumoniae*), furanoeudesma-1,3-diene (*H. influenzae, M. smegmatis*) and *p*-cymene (*H. influenzae, C. neoformans*).

The repetitive biomarkers for the essential oils in class two

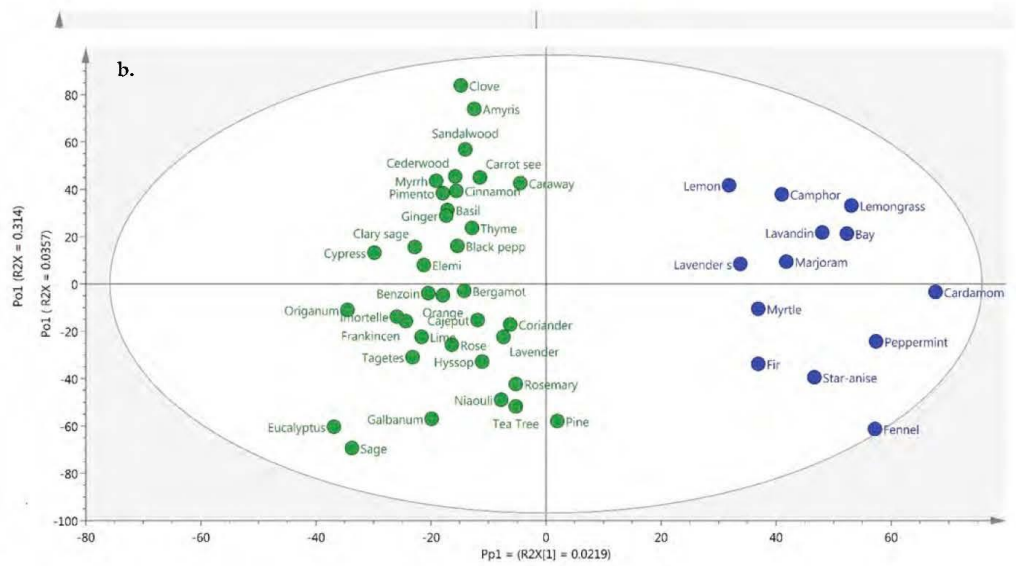


Fig. 2. An OPLS-DA scores scatter plot demonstrating the two classes defined, active or essential oils with noteworthy antimicrobial activity (MIC values ≤ 1.00 mg/ml) (green samples-on the left) and inactive or essential oils with a less or poorer antimicrobial activity (MIC values > 1.00 mg/ml) (blue samples-on the right) essential oils against the pathogen *M. catarrhalis* (a) and *C. neoformans* (b).

demonstrating poor antimicrobial activity include linalyl acetate (*S. aureus, S. pyogenes, H. influenzae*), α-pinene (*S. aureus, H. influenzae, M. catarrhalis, K. pneumoniae*) and β-pinene (*S. aureus, S. agalactiae, S. pneumoniae, S. pyogenes, H. influenzae*).

3.4. Chemometric validation

The accuracy of the chemometric predictions for putative biomarkers of antimicrobial activity was investigated for the compounds identified per respiratory pathogens studied (Table 6).

The incidence of prediction accuracy relating to the chemometric outcomes of this study was 92.3% for active biomarker predictions and 61.9% for inactive biomarker predictions. Against the pathogen, *M. catarrhalis* which showed the strongest chemometric model fit, accuracies in prediction were determined as 100.0% in both classes (active and inactive). Eugenol was predicted as the biomarker responsible for perceived good antimicrobial activity of essential oils against this pathogen. When investigated, eugenol demonstrated an MIC value of 1.00 mg/ml, three dilutions more effective than the compounds α-pinene and *p*-cymene (8.00 mg/ml), determined as responsible for the poor antimicrobial activity of essential oils against this pathogen. Against the pathogen, *C. neoformans* which showed the poorest chemometric model fit, accuracies in prediction were determined as 100% for the active class and 0% for the inactive class with menthone demonstrating similar antimicrobial activity to α-pinene of 1.00 mg/ml.

4. Discussion

4.1. Antimicrobial activity

The essential oils of aromatic plants and spices contain biologically active components with antibacterial and antifungal potential [79]. *Amyris balsamifera* is indicated within aromatherapeutic text for use in respiratory tract infections associated with coughs and colds [8]. Previous studies on the antimicrobial effect of *A. balsamifera* essential oil have been reported using disk diffusion or agar diffusion methodologies against pathogens such as *Candida albicans, S. aureus, E. coli, Pseudomonas aeruginosa* and *K. pneumoniae* [80,81]. The use of disk diffusion assays for antimicrobial testing of essential oils is still widely applied and published; however, this methodology has been fraught with fault and has been deemed by researchers to be an unreliable method for antimicrobial susceptibility testing of essential oils [82,83]. Only two other studies could be identified using *A. balsamifera* essential oil for antimicrobial purposes by means of MIC analysis against the pathogens *S. aureus, Salmonella enteritidis, Pseudomonas aeruginosa, Alternaria alternata, Aspergillus niger* and *Penicillium digitatum* [84,85]. The study by Kloucek et al. [84] made use of a unique methodology in which the antimicrobial activities of essential oil vapours were measured. The results of the study showed *A. balsamifera* essential oil to have antimicrobial activity against *S. aureus* with an MIC of 500 μl/L, while no activity was observed against the other test pathogens. The study conducted by Xiao et al. [85] made use of MIC analysis against *S. aureus* and validated the outcomes via time-kill assay. Results demonstrated *A. balsamifera* essential oil to have a cidal antimicrobial effect against *S. aureus* at 0.125% (*v/v*) following three days of exposure. Other essential oils with broad-spectrum activity include *C. sativum* and *C. zeylanicum*. Both oils have been studied extensively in the literature with results congruent to this study confirming *C. sativum* and *C. zeylanicum* oils to be effective against a wide range of clinically relevant Gram-positive bacteria, Gram-negative bacteria and yeasts [86–96].

It is known that essential oils demonstrate antifungal activity due to their ability to cause damage to fungal cell membranes by binding to the cell wall building blocks called ergosterols [97]. The more lipophilic the substance, the greater the antifungal effect. *Coriandrum sativum* was the most potent essential oil against the pathogen *C. neoformans*.

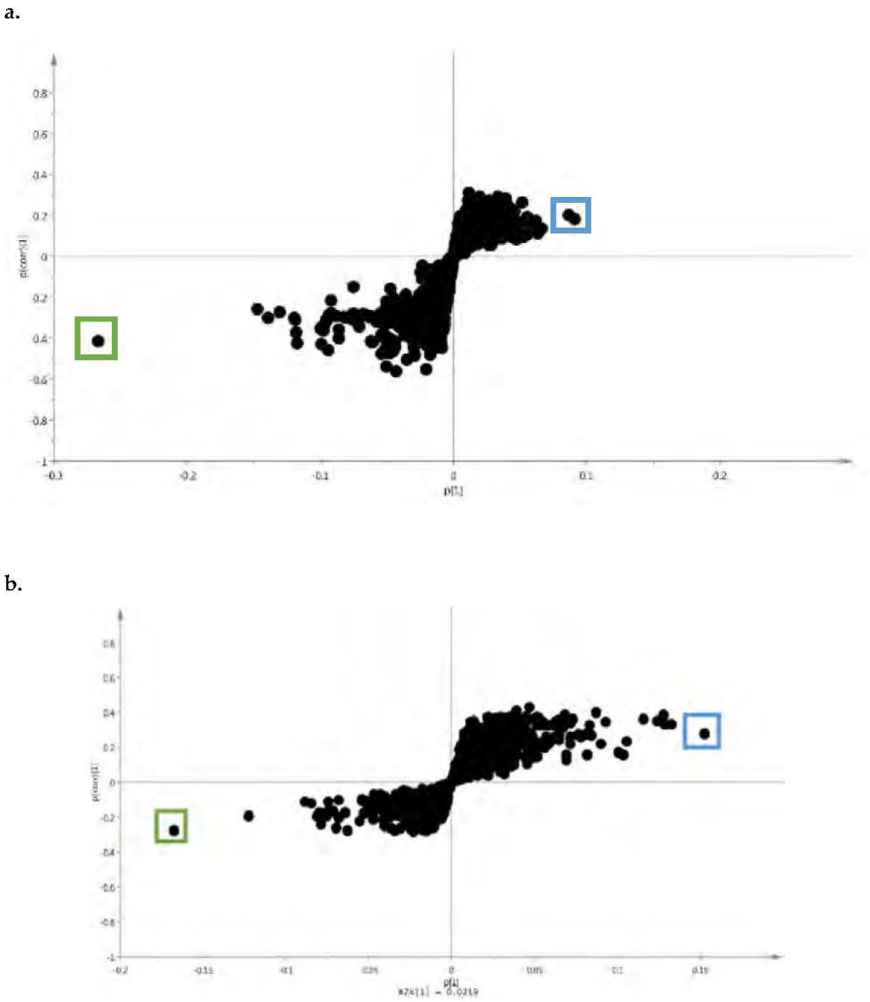


Fig. 3. An OPLS-DA S-plot showing the putative biomarkers responsible for contributing to good activity (bottom left quadrant, green square) and poor activity (top right quadrant, blue square) against the pathogen *M. catarrhalis* (a) and *C. neoformans* (b).

Cryptococcus neoformans causes pathogenesis in immunocompromised hosts resulting in fungal pneumonia and cryptococcosis [29,30]. Interestingly, *C. sativum* essential oil is indicated for the treatment of influenza and broad cases of chest infection [8,10,98]. The result of *C. sativum* against *C. neoformans* is supported by a previous study conducted by Powers et al. [99], in which the MIC values for a common set of 82 essential oils against the pathogens *Aspergillus niger, Candida albicans*, and *C. neoformans* were reported. The essential oil of *C. sativum* showed anti-yeast activity against *C. neoformans* with an MIC value of 80 ppm (where anti-yeast activity is determined for an MIC value < 100 ppm).

Santalum austrocaledonicum essential oil also demonstrated noteworthy antimicrobial effects against the pathogen, *S. pyogenes*. Pneumococcal infections have been identified by the World Health Organization (WHO) as causing the greatest number of respiratory infections among Gram-positive bacteria [100]. *Streptococcus pyogenes* has been identified as the pathogen most responsible for infections of the upper and lower respiratory tract [100]. Sandalwood essential oils, have been commonly cited in the literature as having noteworthy antimicrobial effects against a number of microbial pathogens studies [18,101–103]. Orchard et al. [104], states that the species of sandalwood oil used in this study, *S. austrocaledonicum* shows significantly improved antimicrobial effects to that of *S. album*, when comparing antimicrobial activities. Differences could be observed between the two *Santalum* spp., of which *S. austrocaledonicum* mostly displayed stronger antimicrobial and broad-spectrum activity than that observed by *S. album*. It is proposed that this result is due to differences in chemistry in which the higher content of santalol in *S. austrocaledonicum* lends itself to an improvement in antimicrobial activity for *Santalum* spp. In the study by Orchard et al. [104], *S. austrocaledonicum* shows broad-

Table 4
Identified biomarkers contributing to good antimicrobial activity of essential oils (class 1) tested against selected respiratory tract pathogens.

Pathogen	Ret. time (min)	Mass pair	Identified putative biomarker*
Staphylococcus aureus (ATCC 25924)	32.720	121	Linalool
	21.171	93	*cis*-β-Ocimene
Streptococcus agalactiae (ATCC 55618)	49.834	135	Thymol
Streptococcus pneumoniae (ATCC 49619)	49.772	164	**Eugenol**
Streptococcus pyogenes (ATCC 12344)	49.772	164	**Eugenol**
Mycobacterium smegmatis (ATCC 19420)	49.073	108	Furanoeudesma 1,3-diene
	36.149	94	Santalene
Haemophilus influenzae (ATCC 19418)	49.834	135	Thymol
	22.847	119	*p*-Cymene
	47.942	164	Viridiflorol
	49.073	108	Furanoeudesma 1,3-diene
Klebsiella pneumoniae (ATCC 13883)	49.772	164	**Eugenol**
	21.171	93	*cis*-β-Ocimene
Moraxella catarrhalis (ATCC 23246)	49.772	164	**Eugenol**
Cryptococcus neoformans (ATCC 14116)	12.015	93	α-Pinene

***Bold** – Putative biomarker identified more than twice across pathogens tested.

Table 5
Identified biomarkers contributing to poor antimicrobial activity of essential oils (class 2) tested against selected respiratory tract pathogens.

Pathogen	Ret. time (min)	Mass pair	Identified putative biomarker*
Staphylococcus aureus (ATCC 25924)	15.618	136	**β- Pinene**
	12.015	93	**α-Pinene**
	47.942	164	Viridiflorol
	33.218	93	**Linalyl acetate**
Streptococcus agalactiae (ATCC 55618)	15.618	136	**β- Pinene**
Streptococcus pneumoniae (ATCC 49619)	15.590	93	**β- Pinene**
Streptococcus pyogenes (ATCC 12344)	15.67	136	**β- Pinene**
	33.177	93	**Linalyl acetate**
	18.003	93	Myrcene
	20.138	111	1,8-Cineole
Mycobacterium smegmatis (ATCC 19420)	49.772	164	Eugenol
Haemophilus influenzae (ATCC 19418)	20.137	136	1,8-Cineole
	11.921	93	**α-Pinene**
	15.617	136	**β- Pinene**
	19.793	121	Limonene
	33.176	93	**Linalyl acetate**
Klebsiella pneumoniae (ATCC 13883)	11.921	93	**α-Pinene**
	22.815	119	*p*-Cymene
Moraxella catarrhalis (ATCC 23246)	11.921	93	**α-Pinene**
	22.815	119	*p*-Cymene
Cryptococcus neoformans (ATCC 14116)	30.436	112	Menthone

***Bold** – Putative biomarker identified more than twice across pathogens tested.

spectrum antimicrobial activity with noteworthy effects against the pathogen, *S. aureus* (MIC value of 0.25 mg/ml).

4.2. Chemometric analysis

Research has reported that essential oils predominant in aldehydes or phenols, such as carvacrol, eugenol, or thymol and are characterized by noteworthy antibacterial activity [105–107]. This is because aldehyde and phenol chemical compounds are responsible for the disruption of the cytoplasmic membrane which is the protective armor of the microbial cell [107]. The chemistry of essential oils provides a significant contribution to the antimicrobial effects of essential oils. In a study conducted by Orchard et al. [23], chemometrics was applied to determine the biomarkers responsible for the antimicrobial activity which identified 59 essential oils against 13 pathogens of the skin. Thirty-eight of the essential oils used in the Orchard et al. [23] study were used in this study with similarities in one pathogen investigated, *S. aureus*. The variation modelled that could be related to differentiation of the two classes was very low in Orchard et al. [23], with all models showing predictive variation of less or equal to 5% (R2Xp), while the cumulative variation was less than 10% (R2Xcum). Only two of the models created by Orchard et al. [23], for methicillin-resistant *S. aureus* and *E. coli*, showed a high predictive ability in essential oil activity. These findings are congruent with those of this report and have been attributed to the biological and analytical variation in the dataset [23].

From the data obtained in this study, α-pinene, β-pinene and linalyl acetate were identified as the biomarkers most frequently responsible for a poor antimicrobial effect while eugenol was identified as the biomarker most frequently responsible for a broad-spectrum antimicrobial activity. These findings are congruent with previous studies [21,23]. Interestingly, the putative biomarkers responsible for perceived poor antibacterial activity were responsible for good antifungal activity according to OPLS-DA S-plot data. The compound α-pinene was identified as a putative biomarker responsible for poorer antibacterial effects against four of the eight bacterial pathogens investigated while responsible for good antifungal activity against the pathogen *C. neoformans*. In a study conducted by Ebrahimabadi et al. [108], the chemical components of the essential oil samples of *M. communis* (myrtle) were identified qualitatively and quantitatively using GC/FID/Mass spectrometry system, and evaluated in conjunction with the antimicrobial activity of these samples using chemometrics methods. The results showed that α-pinene, 1,8-cineole, β-pinene and limonene were the highest contributors to the antimicrobial activity of *M. communis* essential oil against *C. albicans*. The antifungal potential of α-pinene is further demonstrated in a study by Rivas da Silva et al. [109], where (+)-α-pinene showed the best MIC inhibition results against *C. neoformans* with a value of 117 μg/ml. Further research has shown α-pinene to have anti-*Cryptococcal* activity as low as 70–78 μg/ml [99,110,111]. The poorer antibacterial effects of α-pinene has been previously proven in literature where it is noted that essential oils containing terpene hydrocarbons are usually associated with poorer antibacterial activity [112,113]. Furthermore, *in vitro* studies indicate that terpenes show ineffective antimicrobial activity when used as singular compounds [112].

The validation of the chemometric analysis demonstrated that the majority of models accurately predicted the compounds associated with good antimicrobial activity against the nine tested pathogens. The compounds with the greatest antimicrobial effect were determined as furanoeudesma-1,3-diene against *H. influenzae* (MIC value of 0.10 mg/ml) and *M. smegmatis* (MIC value of 0.18 mg/ml); and viridiflorol against the pathogen *H. influenzae* with an MIC value of 0.08 mg/ml. Furanoeudesma-1,3-diene is a colourless, unstable liquid with an intensive aroma which rapidly auto-oxidizes in air at room temperature to leave a resinous residue [114]. Furanoeudesma-1,3-diene is a major constituent of *Commiphora* essential oils including *C. molmol*. No previous studies could be identified to prove the antimicrobial activity of furanoeudesma-1,3-diene against either *H. influenzae* or *M. smegmatis*. Viridiflorol is a sesquiterpenoid commonly isolated from essential oils of *Melaleuca quinquenervia* (broad-leaved paperbark), *M. alternifolia*, *Salvia* species and *Mentha* species [115]. No previous studies could be identified for the antimicrobial activity of viridiflorol against *H. influenzae*, however, studies investigating the antimicrobial effects of this compound against pathogens such as *S. aureus* and *M. tuberculosis* show promising antimicrobial effects [115,116].

Greater inaccuracies in prediction were determined for the inactive class . The compounds viridiflorol and 1,8-cineole demonstrated noteworthy antimicrobial activity against the pathogens associated with

Table 6
The Minimum Inhibitory Concentration (MIC in mg/ml (n 3)) antimicrobial activity of the putative biomarkers identified for the pathogens investigated correlated to chemometric prediction accuracies.

Pathogen	Antimicrobial validation					Chemometrics
	Active biomarker	MIC	Accuracy	Inactive biomarker	MIC	Accuracy	$Q^2(cum)$[b]
Cryptococcus neoformans (ATCC 14116)	α-Pinene	1.00	100%	Menthone	1.00	0%	-0.097
Haemophilus influenzae (ATCC 19418)	Thymol	0.50	100%	1,8-Cineole	0.50	20%	0.017
	p-Cymene	0.25		α-Pinene	8.00		
	Viridiflorol	**0.08**		β- Pinene	1.00		
	Furanoeudesma 1,3-diene	**0.10**		Limonene	1.00		
				Linalyl acetate	1.00		
Klebsiella pneumoniae (ATCC 13883)	Eugenol	1.00	100%	α-Pinene	8.00	100%	-0.158
	cis-β-Ocimene	1.00		*p*-Cymene	8.00		
Moraxella catarrhalis (ATCC 23246)	Eugenol	1.00	100%	α-Pinene	8.00	100%	0.279
				p-Cymene	8.00		
Mycobacterium smegmatis (ATCC 19420)	Furanoeudes-ma 1,3-diene	0.18	100%	Eugenol	1.00	0%	-0.012
	Santalene	[a]n.d.					
Staphylococcus aureus (ATCC 25924)	Linalool	1.00	50%	β- Pinene	2.00	75%	0.061
				α-Pinene	8.00		
	cis-β-Ocimene	2.00		Viridiflorol	0.32		
				Linalyl acetate	2.00		
Streptococcus agalactiae (ATCC 55618)	Thymol	0.25	100%	β- Pinene	2.00	100%	-0.054
Streptococcus pneumoniae (ATCC 49619)	Eugenol	1.00	100%	β- Pinene	1.00	0%	0.139
				β- Pinene	4.00		
Streptococcus pyogenes (ATCC 12344)	Eugenol	1.00	100%	Linalyl acetate	2.00	100%	0.100
				Myrcene	2.00		
				1,8-Cineole	2.00		

Bold - clinically relevant antimicrobial activity
[a] n.d. – not determined due to lack of availability.
[b] Ability to predict future outcomes.

poor antimicrobial effect predictions. Viridiflorol was predicted as the biomarker of essential oils responsible for causing poor antimicrobial effects against the pathogen *S. aureus*. The results of this study determined an MIC value of 0.32 mg/ml for viridiflorol against *S. aureus*, the lowest MIC determined against this pathogen. A study conducted by Gilabert et al. [116] aimed to determine the ability of ten *Lepidozia chordulifera* terpenoids, including viridiflorol, to control bacterial growth and biofilm formation of *S. aureus*. Among the compounds tested, only viridiflorol was able to inhibit bacterial growth and biofilm formation by 40% against *S. aureus* at 50 μg/ml. These findings support the outcome of this study in that viridiflorol is an effective antibacterial compound against the pathogen *S. aureus*. The compound 1,8-cineole (eucalyptol), is a monoterpene cyclic ether found as the major component of essential oils isolated from *Eucalyptus globulus* leaves as well as in *Rosmarinus officinalis, Salvia officinalis* and *Mentha* species [117]. The pharmacological effects of 1,8-cineole have been well documented with mucolytic, broncholytic, and anti-inflammatory properties identified [117]. The results of this study determined an MIC value of 0.50 mg/ml for 1,8-cineole against *H. influenzae*. A study conducted by Yang et al. [118] aimed to determine the antibacterial activity and mode of action of the essential oil of *Artemisia capillaris* and its constituents, including 1,8-cineole against respiratory tract infection-causing pathogens such as *H. influenzae*. The compound 1,8-cineole demonstrated noteworthy antimicrobial activity against *H. influenzae* with an MIC value of 0.098 mg/ml.

Despite these findings, the chemometric models show a significantly high level of predictability of interactions. The model fit ($Q^2(cum)^2$) for half of the models created showed negative ability to predict future outcomes. Despite this, the chemometric models were in the majority accurate in prediction. This outcome may be as a result of the complexity in the ability to predict compounds associated to good or poor behavior in multifarious products such as essential oils. It is known that the major chemical constituents of an essential oil are often considered responsible for the perceived antimicrobial activity, however, this study demonstrates the potential for minor components to provide significance in the perceived antimicrobial activity of essential oils.

5. Conclusions

This study is the first to report on the antimicrobial potential of commercial essential oils such as *A. balsamifera*, *S. austrocaledonicum* and *C. sativum* among others for use in respiratory tract infections against a large variety of pathogenic micro-organisms. The effect of essential oils against pathogens most commonly isolated in bacterial respiratory tract infections such as *S. pyogenes* and *S. pneumoniae* have been reported here for the first time. The essential oils that displayed broad-spectrum antimicrobial effect are; *A. balsamifera*, *C. sativum* and *S. austrocaledonicum*. Eugenol was identified as the most frequently reoccurring biomarker that contributes to broad-spectrum noteworthy antimicrobial activity, while linalyl acetate, α-pinene and β-pinene were frequently responsible for poor antimicrobial activity. The ability of chemometrics to accurately predict the active and inactive biomarkers of essential oils against the pathogens assessed were mostly confirmed by this study with an accurate prediction of 92.3% for active biomarkers and 61.90% for inactive biomarkers. The effect of essential oil compounds viridiflorol and furanoeudesma-1,3-diene against the pathogen *H. influenzae* have been reported here for the first time. Outcomes of this study suggest that perceived biological activity of an essential oil may be more closely related to the activity of a single chemical constituent, however, the potential for interactions between essential oil chemical compounds predicted for perceived good antimicrobial effect should be investigated further.

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Credit author statement

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relationships of essential oils used in respiratory tract infections" by authors Stephanie Leigh-de Rapper, Sidonie Yankam Tankeu, Guy Kamatou, Alvaro Viljoen and Sandy van Vuuren. All authors have contributed towards the manuscript and support the submission thereof and have no conflicting interests. Furthermore, all authors have contributed towards the revision.

Declaration of Competing Interest

The authors declare that they have no known competing interests.

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CHAPTER 3:

DETERMINING AND OPTIMIZING SYNERGY AMONG ESSENTIAL OIL COMBINATIONS



3. Introduction

Following the investigation into the antimicrobial activity of individual essential oils that are commonly cited for use in the respiratory tract against microbial reference strains, an investigation into the combined pharmacological effect of essential oils was to be considered. Aromatic medicine predominantly applies essential oils in multiple combinations, as aromatherapy is based on the practice of blending oils to achieve an enhanced therapeutic effect (van Vuuren and Viljoen, 2011). The rationale for combining essential oils includes creating blends that will enhance antimicrobial activity, or provide antimicrobial effect with anti-inflammatory activity, or an antimicrobial and psychological effect, or enhanced pharmacological effect with reduced toxicity (Lawless, 1995, Shealy, 1998, Hili, 2001, Sellar, 2005, Clarke, 2009, Buckle, 2014, Curtis et al., 2016, Orchard, 2019). The review of the aromatherapeutic literature identified 369 combinations used in the respiratory tract for varying enhanced antimicrobial, anti-inflammatory and non-toxic effects (Leigh-de Rapper et al., 2021). The concern regarding these combinations is that available data on the antimicrobial interaction of the intended therapeutic combinations is lacking. This study addressed this deficiency of information by evaluating the identified essential oil combinations against the micro-organisms selected in Chapter 2. The outcome of the combination study is reported in **Article 3**.

3.1. Synergy optimization

Studies documenting the combined use of essential oils for antimicrobial purposes predominantly makes use of these essential oils in equal concentrations (Kemegne et al., 2021). However, where essential oils have been studied in varied ratios it has been determined that the antimicrobial benefit of the combination is not limited to the property of a single oil, or when placed in equal concentration, but depends on the specific ratios in which they are combined (Cassella et al., 2002, Orchard et al., 2018a, Kemegne et al., 2021). The assessment of essential oil combinations for synergy optimization is complex due to the large number of variations that exist; however, computer-aided software provides the means to review these combinations at fixed doses to predict synergy at infinite ratios. The use of computer-aided software has reduced the cost burden associated with drug design and research and has been formally recognized by the US FDA for clinical trial research (Sabe et al., 2021). Computer-aided drug design is an indispensable tool in the pharmaceutical industry (Liao et al., 2011).

3.1.1. Current essential oil antimicrobial research using computer-aided software

Design of Experiments (DoE) software is computer-aided software currently employed within industries such as pharmacy, chemistry and engineering (Thakkar et al., 2021, Hathout and Saharan, 2022, Nainwal et al., 2022). It is used in the industry as a means of optimizing experimental outcomes using a limited amount of experiments and resources (Carlson, 2001). A study conducted by De Rapper (2013) aimed to identify the antimicrobial effects of a number of varying essential oils in combination with *Lavandula angustifolia* (lavender) essential oil in triplicate combinations against *Candida albicans*. The study used MODDE 9.1® software for antimicrobial predictions. Three varying combinations of essential oils were investigated by comparing the MIC data obtained from the various combinations against the data gained from the individual MIC values of the essential oils under investigation (Carlson, 2001). For the combinations investigated it was determined that no single essential oil in the combination was responsible for an enhanced antimicrobial effect as the MODDE 9.1® software did not vary considerably even when essential oil volumes did. Limitations to the effectiveness of this software for antimicrobial purposes is the inability for the software to predict the optimal use of essential oil combinations where MIC values are similar and the restriction on the number of samples available for combination predictions.

3.1.2. SynergyFinder predictive software for potential use within essential oil research

SynergyFinder is a computer-based program for the automation and quantitation of synergism and antagonism, that applies commonly-utilized reference models such as, the Highest Single Agent (HSA) model (Berenbaum, 1989), the Loewe additivity model (Loewe, 1953), the Bliss independence model (Bliss, 1939), and the Zero Interaction Potency (ZIP) model (Yadav et al., 2015) for prediction. Using these reference models, SynergyFinder then implements algorithms to calculate synergy scores based on dose-response data, which are then used to identify the ratio of essential oil in each combination that provides the greatest synergistic antimicrobial response. The use of this software has been published in over 100 scientific papers ranging from oncology to drug design and drug combination studies. The use of this type of software within the field of antimicrobial research is novel, with limited data available to validate its potential for use in this field of research (Nguyen et al., 2020, Maugeri et al., 2021). This study is the first to optimize essential oil combinations using SynergyFinder predictive software (**Article 4**).

Article 3 - Original Article on the combined effects of essential oils in the management of respiratory infections

At the time of this thesis, this article had been published. This article is shown as it appears in publication.

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Article

Essential Oil Blends: The Potential of Combined Use for Respiratory Tract Infections

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Abstract: This study investigated the potential efficacy of 369 commercial essential oil combinations for antimicrobial, anti-toxic and anti-inflammatory activity with the aim of identifying synergy among essential oils commonly used in combination by aromatherapists for respiratory purposes. Essential oil combinations were assessed for their antimicrobial activities using a panel of Gram-positive, Gram-negative, and yeast strains associated with respiratory tract infections. The antimicrobial activity was measured by determining the minimal inhibitory concentration (MIC) of microbial growth. The fractional inhibitory concentration index (ΣFIC) was calculated to determine the antimicrobial interactions between the essential oils in the combination. The toxicity of the essential oil combinations was tested *in vitro* using the brine shrimp lethality assay, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on RAW 264.7 mouse macrophage cells and A549 lung cancer cell lines. In addition, an inflammatory response was evaluated measuring nitric oxide production. The essential oils, when in combination, demonstrated an increased antimicrobial effect, a reduction in toxicity and provided improved anti-inflammatory outcomes. Five distinct combinations [*Cupressus sempervirens* (cypress) in combination with *Melaleuca alternifolia* (tea tree), *Hyssopus officinalis* (hyssop) in combination with *Rosmarinus officinalis* (rosemary), *Origanum marjorana* (marjoram) in combination with *M. alternifolia*, *Myrtus communis* (myrtle) in combination with *M. alternifolia* and *Origanum vulgare* (origanum) in combination with *M. alternifolia*] were found to be the most promising, demonstrating antimicrobial activity, reduced cytotoxicity and improved anti-inflammatory effects. With the increased prevalence of respiratory tract infections and the growing antimicrobial resistance development associated with antimicrobial treatments, this study provides a promising complementary alternative for the appropriate use of a selection of essential oil combinations for use in the respiratory tract.

Keywords: antimicrobial; anti-inflammatory; inhalation; essential oils; combinations; synergy; toxicity; respiratory tract

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1. Introduction

Antimicrobial resistance, partly a consequence of inappropriate antibiotic use, has been continuously recorded globally and is considered one of the greatest challenges to global public health [1]. *Streptococcus pneumoniae*, a commonly isolated pathogen of the respiratory tract, has developed and spread resistance to antibiotics such as penicillins, with penicillin resistance often correlating to resistance to other additional antibiotics such as macrolides and tetracyclines [2,3]. A possible solution to growing antimicrobial resistance is the use of alternative and complimentary therapies which have been shown to elicit antimicrobial effects as well as holistically treat symptoms often caused by infections. The emergence of new antibacterial agents based on natural products is a priority in the field

of scientific research with studies demonstrating the potential of these products against pathogens of multidrug resistant causes [4,5].

Essential oils are comprised of volatile, aromatic and complex chemical compounds such as alcohols, aldehydes, esters, ethers, ketones, phenols and terpenes. These essential oils are distilled from plant parts and commonly employed in aromatherapy. Within the scientific literature, essential oils have been extensively studied with relevance to respiratory conditions. A plethora of studies are available where essential oils have been studies for antimicrobial purposes as well as for inhalation in traditional practices and in maintaining basic health conditions [6–11]. Although there has been a surge in interest and encouraged direction in the field of essential oil research, much of the research published focuses on identifying the potential of a single essential oil [12]. Within aroma therapeutic use, however, essential oils are predominantly used in multiple combinations, as aromatherapy is based on the practice of combining multiple essential oils to achieve an enhanced therapeutic effect [13]. Insights into the use of complementary and alternative therapies, including aromatherapy, within developed countries has identified that $25 billion is spent annually by people actively seeking CAM treatments to prevent or treat ongoing infections [14]. This growth in the use of CAM treatments requires an equal growth in research for the evaluation of these products and practices for quality, safety and efficacy.

The aroma-therapeutic literature notes extensively the use of essential oils in combination for improved antimicrobial, anti-oxidative, anti-inflammatory, as well as antihistaminic effects [15]. Previous studies have demonstrated the therapeutic potential of some commercial and indigenous essential oils when tested in combination [16–20]. However, a lack of data still exists for the antimicrobial efficacy of a large number of commonly applied commercial essential oils used in combination. Inflammation is a physiological response by a host to regulate the body's reaction to infections, injury or toxins [21]. Acute inflammation is initiated by tissue-resident innate immune cells that recognize pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) caused by infection or cell injury [22]. In the case of an infection caused by microbial invasion, initial recognition of infection is mediated by tissue-resident macrophages and mast cells. These cells induce inflammation through the production of a variety of inflammatory mediators, including chemokines, pro-inflammatory cytokines, vasoactive amines, eicosanoids and products of proteolytic cascades to enhance inflammatory signals and recruit more immune cells [23]. The release of cytokines, such as interferon-γ (INF-γ), TNF-α, and interleukin-1 (IL-1), as well as exposure to microbial products such as LPS further stimulate macrophages to produce nitric oxide (NO) [24]. The immediate effect of these mediators is to cause local vasodilation as a means of providing plasma proteins and leukocytes (mostly neutrophils), normally held in circulation, to the extravascular tissues at the site of infection. Once at the site of infection, neutrophils become activated either by direct contact with pathogens or through the actions of cytokines secreted by tissue-resident cells [23]. The neutrophils then attempt to kill the invading pathogen by releasing the toxic contents of their granules [25]. The production of NO by macrophages further contributes to the elimination of invading pathogens by inducing the cytotoxic action of these macrophages. When the inflammatory response has resulted in the elimination of the infectious agents, a resolution and repair phase commences to reduce inflammation [26]. If the inflammatory response does not result in the elimination of the causative pathogen, the inflammatory process persists, leading to a chronic state of inflammation. The characteristics of this inflammatory state differ depending on the effector class of the T cells that are present [23]. The further influx of immune cells and increased inflammatory response can cause a reduction in gas exchange due to fluid accumulation, and further result in damage to the lungs, resulting in severe respiratory infection [27].

Essential oils have been well studied, and demonstrate anti-inflammatory effects [28–31]. Essential oils with anti-inflammatory activity have been shown to reduce swelling and edema associated with respiratory infections, thus reducing symptoms such as wheezing, congestion and difficulty in breathing. Despite this, essential oils are commonly used in

combination in aromatherapy, and very little literature exists to support this application of essential oils for anti-inflammatory effects. Given the importance of the inflammatory process in infections of the respiratory tract, it is important to determine the potential for essential oils used in combination to influence this infection process.

Essential oils have also been associated with high levels of toxicity, especially when compared to other natural products [15]. Essential oils are registered on the United States Food and Drug Authority (US, FDA) list of 'generally recognized as safe' (GRAS) products as individual agents but not as combinations [15]. The activity between blended essential oils has the potential to increase toxicity or suppress these effects. Small doses of essential oils are capable of eliciting toxic effects, especially when inhaled [32]. Despite these findings, essential oils remain the most popular form of complementary medicine and continue to be used in combination via dermal or inhalation application. It is a result of this lack of data to substantiate the use of essential oils in combination for antimicrobial and anti-inflammatory effects, as well as the increased risk of toxicity associated with essential use when combined, that further research is needed to confirm the therapeutic potential for use of essential oils via the respiratory tract. This study, therefore, aims to determine the antimicrobial, anti-inflammatory and toxicity levels of commercial essential oil combinations commonly applied in aromatherapy for respiratory tract infections.

2. Results and Discussion

2.1. Antimicrobial Analysis of Essential Oils

The MIC values of the 49 individual commercial essential oils were investigated in our previous study [33]. Of the 369 combinations investigated (Supplementary Table S1), 41.6% displayed noteworthy activity against the nine respiratory pathogens investigated. Of the combinations determined to be noteworthy in antimicrobial effect, 27.3% elicited strongly noteworthy antimicrobial effects of less than 0.50 mg/mL, while 72.3% of combinations displayed moderately noteworthy effects between 0.50 and 1.00 mg/mL. Figure 1 summarizes the percentage of essential oils that were found to have a noteworthy antimicrobial activity alone (a) and in combination (b).

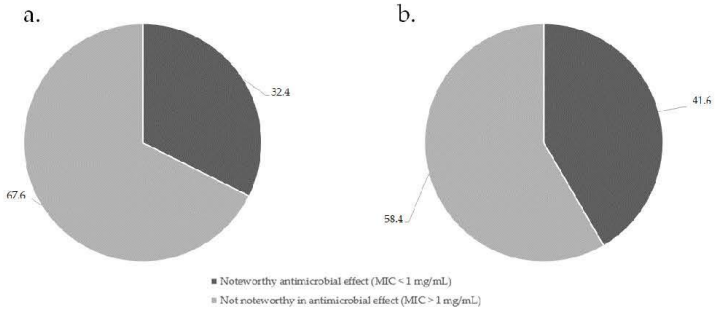


Figure 1. Summary of noteworthy essential oil antimicrobial activity alone (**a**) and when in combination (**b**).

From these results, it is clear that the essential oils show the potential to demonstrate improved antimicrobial efficacy when in combination. As the focus was on finding the combinations with the highest antimicrobial efficacy, the best results observed for the 369 investigated combinations (1:1) against respiratory tract pathogens are summarized in Table 1.

Table 1. The mean MIC ($n = 3$) with standard deviation in brackets and ΣFIC values of the essential oil combinations investigated against pathogens of the respiratory tract.

Essential Oil Combinations (Common Name in Brackets)		Mean MIC Value (mg/mL) ($n = 3$) and ΣFIC																	
		Staphylococcus aureus (ATCC 25924)		*Streptococcus agalactiae* (ATCC 55618)		*Streptococcus pneumoniae* (ATCC 49619)		*Streptococcus pyogenes* (ATCC 12344)		*Mycobacterium smegmatis* (ATCC 19420)		*Haemophilus influenzae* (ATCC 19418)		*Klebsiella pneumoniae* (ATCC 13883)		*Moraxella catarrhalis* (ATCC 23246)		*Cryptococcus neoformans* (ATCC 14116)	
Essential Oil 1	**Essential Oil 2**	MIC *	ΣFIC **	MIC *	ΣFIC **	MIC *	ΣFIC **	MIC *	ΣFIC **	MIC *	ΣFIC **	MIC *	ΣFIC **	MIC *	ΣFIC **	MIC *	ΣFIC **	MIC *	ΣFIC **
Amyris balsamifera (amyris)	*Styrax benzoin* (benzoin)	**0.25 (±0.00)**	***0.28***	**0.25 (±0.00)**	***0.31***	**0.75 (±0.35)**	*3.09*	**0.19 (±0.00)**	***0.31***	2.00 (±0.00)	1.25	**0.13 (±0.00)**	*0.53*	1.50 (±0.71)	*0.75*	**0.50 (±0.00)**	***0.38***	**0.50 (±0.00)**	1.17
Cinnamomum zeylanicum (cinnamon)	*Elettaria cardamomum* (cardamom)	**0.50 (±0.00)**	***0.50***	**1.00 (±0.00)**	1.25	2.00 (±0.00)	*1.00*	**0.75 (±0.35)**	1.69	**1.00 (±0.00)**	***0.31***	2.00 (±0.00)	1.67	**0.50 (±0.00)**	***0.50***	2.00 (±0.00)	2.50	**0.13 (±0.00)**	***0.13***
	Zingiber officinale (ginger)	**0.50 (±0.00)**	***0.50***	**0.50 (±0.00)**	*0.75*	**1.00 (±0.00)**	***0.50***	**0.19 (±0.00)**	***0.47***	4.00 (±0.00)	2.00	**0.50 (±0.00)**	***0.42***	**0.50 (±0.00)**	***0.31***	2.00 (±0.00)	3.00	**0.13 (±0.00)**	***0.21***
Citrus bergamia (bergamot)	*Cupressus sempervirens* (cypress)	4.00 (±0.00)	*0.92*	2.00 (±0.00)	*1.00*	**1.00 (±0.00)**	***0.31***	6.00 (±2.83)	2.00	**1.00 (±0.00)**	***0.25***	1.50 (±0.71)	***0.50***	2.00 (±0.00)	*0.83*	2.00 (±0.00)	***0.25***	**1.00 (±0.00)**	1.50
	Rosmarinus officinalis (rosemary)	2.00 (±0.00)	***0.38***	**1.00 (±0.00)**	***0.50***	2.00 (±0.00)	*1.00*	8.00 (±0.00)	*5.33*	2.00 (±0.00)	***0.50***	**0.75 (±0.35)**	***0.17***	**0.75 (±0.35)**	***0.31***	**1.00 (±0.00)**	***0.31***	1.50 (±0.71)	*3.75*
Citrus limon (lemon)	*Santalum austrocaledonicum* (sandalwood)	**0.38 (±0.18)**	***0.27***	**0.13 (±0.00)**	***0.28***	**0.25 (±0.00)**	***0.13***	**0.19 (±0.00)**	1.55	2.00 (±0.00)	1.58	**0.50 (±0.00)**	*2.03*	4.00 (±0.00)	*0.92*	**1.00 (±0.00)**	2.50	**0.06 (±0.00)**	***0.19***
Coriandrum sativum (coriander)	*Cinnamomum zeylanicum* (cinnamon)	**0.50 (±0.00)**	*0.75*	**0.13 (±0.00)**	***0.19***	**1.00 (±0.00)**	*0.75*	**0.38 (±0.18)**	1.50	**0.50 (±0.00)**	***0.25***	**0.50 (±0.00)**	*1.00*	**1.00 (±0.00)**	1.50	2.00 (±0.00)	*2.67*	**0.13 (±0.00)**	*2.08*
Cupressus sempervirens (cypress)	*Hyssopus officinalis* (hyssop)	2.00 (±0.00)	*0.83*	2.00 (±0.00)	*1.00*	**1.00 (±0.00)**	***0.31***	1.50 (±0.71)	*1.00*	1.50 (±0.71)	*0.38*	2.00 (±0.00)	***0.46***	**1.00 (±0.00)**	*0.50*	2.00 (±0.00)	*0.63*	**0.50 (±0.00)**	2.50
	Lavandula angustifolia (lavender)	2.00 (±0.00)	*1.33*	2.00 (±0.00)	*1.00*	1.50 (±0.71)	*0.84*	8.00 (±0.00)	*2.33*	2.00 (±0.00)	*0.75*	4.00 (±0.00)	*1.33*	**1.00 (±0.00)**	*0.58*	2.00 (±0.00)	*0.63*	**0.13 (±0.00)**	***0.19***
	Melaleuca alternifolia (tea tree)	4.00 (±0.00)	*1.17*	**1.00 (±0.00)**	*0.58*	2.00 (±0.00)	*0.63*	1.50 (±0.71)	*0.75*	**1.00 (±0.00)**	*0.25*	3.00 (±1.41)	*1.50*	4.00 (±0.00)	*1.50*	2.00 (±0.00)	*0.63*	**0.09 (±0.00)**	***0.16***
	Salvia sclarea (clary sage)	2.00 (±0.00)	***0.46***	2.00 (±0.00)	*1.00*	2.00 (±0.00)	***0.25***	4.00 (±0.00)	*1.33*	2.00 (±0.00)	***0.50***	2.00 (±0.00)	*0.67*	**1.00 (±0.00)**	***0.31***	2.00 (±0.00)	*0.25*	**0.25 (±0.00)**	*1.25*
Elettaria cardamomum (cardamom)	*Coriandrum sativum* (coriander)	**0.50 (±0.00)**	*0.75*	**0.25 (±0.00)**	***0.19***	**0.50 (±0.00)**	***0.38***	**0.75 (±0.35)**	*1.69*	**1.00 (±0.00)**	***0.31***	**0.50 (±0.00)**	*0.75*	**1.00 (±0.00)**	*1.50*	2.00 (±0.00)	*1.17*	**0.13 (±0.00)**	*2.04*
Eucalyptus globulus (eucalyptus)	*Cymbopogon citratus* (lemongrass)	**1.00 (±0.00)**	*0.63*	**1.00 (±0.00)**	***0.50***	2.00 (±0.00)	*1.67*	**0.13 (±0.00)**	***0.20***	**1.00 (±0.00)**	***0.38***	3.00 (±1.41)	*1.50*	8.00 (±0.00)	*2.50*	2.00 (±0.00)	*1.00*	**0.06 (±0.00)**	***0.04***
Ferula galbaniflua (galbanum)	*Zingiber officinale* (ginger)	2.00 (±0.00)	*1.33*	2.00 (±0.00)	*1.50*	2.00 (±0.00)	*1.00*	**0.25 (±0.00)**	***0.21***	2.00 (±0.00)	*0.67*	8.00 (±0.00)	*1.83*	**1.00 (±0.00)**	***0.38***	2.00 (±0.00)	*1.50*	**0.25 (±0.00)**	*0.58*
Helichrysum italicum (immortelle)	*Lavandula angustifolia* (lavender)	2.00 (±0.00)	*1.25*	**1.00 (±0.00)**	***0.50***	2.00 (±0.00)	*1.50*	**0.75 (±0.35)**	***0.34***	2.00 (±0.00)	*1.00*	**1.00 (±0.00)**	***0.29***	**1.00 (±0.00)**	***0.42***	3.00 (±1.41)	*1.75*	**0.38 (±0.18)**	*1.69*
	Lavandula spica (lavender spike)	**1.00 (±0.00)**	*0.63*	**1.00 (±0.00)**	***0.50***	2.00 (±0.00)	*1.17*	**0.50 (±0.00)**	***0.25***	2.00 (±0.00)	*0.75*	6.00 (±2.83)	*1.50*	**0.75 (±0.35)**	***0.31***	2.00 (±0.00)	*1.67*	**0.50 (±0.00)**	*2.17*

Table 1. *Cont.*

Essential Oil Combinations (Common Name in Brackets)		Mean MIC Value (mg/mL) ($n = 3$) and ΣFIC																	
		Staphylococcus aureus		*Streptococcus agalactiae*		*Streptococcus pneumoniae*		*Streptococcus pyogenes*		*Mycobacterium smegmatis*		*Haemophilus influenzae*		*Klebsiella pneumoniae*		*Moraxella catarrhalis*		*Cryptococcus neoformans*	
		(ATCC 25924)		(ATCC 55618)		(ATCC 49619)		(ATCC 12344)		(ATCC 19420)		(ATCC 19418)		(ATCC 13883)		(ATCC 23246)		(ATCC 14116)	
Essential Oil 1	**Essential Oil 2**	**MIC ***	**ΣFIC ****	**MIC ***	**ΣFIC ****	**MIC ***	**ΣFIC ****	**MIC ***	**ΣFIC ****	**MIC ***	**ΣFIC ****	**MIC ***	**ΣFIC ****	**MIC ***	**ΣFIC ****	**MIC ***	**ΣFIC ****	**MIC ***	**ΣFIC ****
Hyssopus officinalis (hyssop)	*Rosmarinus officinalis* (rosemary)	2.00 (±0.00)	0.75	2.00 (±0.00)	1.00	3.00 (±1.41)	1.50	**1.00 (±0.00)**	1.00	1.50 (±0.71)	0.38	4.00 (±0.00)	**0.50**	4.00 (±0.00)	2.00	2.00 (±0.00)	1.00	**0.09 (±0.00)**	0.56
Laurus nobilis (bay)	*Eucalyptus globulus* (eucalyptus)	**1.00 (±0.00)**	0.63	4.00 (±0.00)	2.00	8.00 (±0.00)	3.67	**0.63 (±0.04)**	**0.26**	2.00 (±0.00)	**0.50**	**1.00 (±0.00)**	**0.29**	**1.00 (±0.00)**	0.58	2.00 (±0.00)	0.63	**0.19 (±0.00)**	**0.12**
Lavandula angustifolia (lavender)	*Citrus aurantifolia* (lime)	3.00 (±1.41)	1.88	2.00 (±0.00)	1.00	2.00 (±0.00)	1.50	2.00 (±0.00)	0.75	**1.00 (±0.00)**	**0.38**	2.00 (±0.00)	**0.46**	**1.00 (±0.00)**	0.58	2.00 (±0.00)	1.00	**0.25 (±0.00)**	**0.29**
Myrtus communis (myrtle)	*Melaleuca alternifolia* (tea tree)	2.00 (±0.00)	**0.50**	**1.00 (±0.00)**	0.58	**1.00 (±0.00)**	**0.50**	2.00 (±0.00)	0.79	2.00 (±0.00)	**0.42**	4.00 (±0.00)	1.67	**1.00 (±0.00)**	**0.38**	**1.00 (±0.00)**	**0.50**	**0.25 (±0.00)**	**0.23**
Ocimum basilicum (basil)		**1.00 (±0.00)**	1.13	2.00 (±0.00)	1.67	2.00 (±0.00)	1.00	**0.63 (±0.00)**	**0.42**	**1.00 (±0.00)**	**0.25**	**0.75 (±0.35)**	**0.31**	1.50 (±0.71)	0.94	2.00 (±0.00)	1.50	**0.13 (±0.00)**	**0.33**
Origanum marjorana (marjoram)		2.00 (±0.00)	**0.38**	**1.00 (±0.00)**	0.58	1.50 (±0.71)	0.75	2.00 (±0.00)	1.67	3.00 (±1.41)	0.88	4.00 (±0.00)	2.00	**1.00 (±0.00)**	**0.38**	**1.00 (±0.00)**	**0.50**	**0.25 (±0.00)**	**0.23**
Origanum vulgare (origanum)		2.00 (±0.00)	1.25	**1.00 (±0.00)**	0.58	**1.00 (±0.00)**	**0.38**	2.00 (±0.00)	1.67	2.00 (±0.00)	**0.50**	4.00 (±0.00)	2.00	**1.00 (±0.00)**	**0.38**	3.00 (±1.41)	1.50	**0.50 (±0.00)**	0.58
Salvia sclarea (clary sage)	*Citrus aurantifolia* (lime)	4.00 (±0.00)	0.75	2.00 (±0.00)	1.00	2.00 (±0.00)	0.63	2.00 (±0.00)	0.83	1.50 (±0.71)	**0.38**	1.50 (±0.71)	**0.34**	8.00 (±0.00)	2.50	2.00 (±0.00)	0.63	**0.19 (±0.00)**	0.88
	Citrus bergamia (bergamot)	8.00 (±0.00)	1.00	**1.00 (±0.00)**	**0.50**	4.00 (±0.00)	1.25	8.00 (±0.00)	2.67	1.50 (±0.71)	**0.38**	1.50 (±0.71)	**0.50**	8.00 (±0.00)	1.83	2.00 (±0.00)	**0.25**	**0.13 (±0.00)**	0.56
	Lavandula burnati (lavandin)	**1.00 (±0.00)**	**0.40**	2.00 (±0.00)	1.00	2.00 (±0.00)	1.13	2.00 (±0.00)	1.33	2.00 (±0.00)	0.75	2.00 (±0.00)	0.83	2.00 (±0.00)	1.13	2.00 (±0.00)	0.63	**0.19 (±0.00)**	0.81
Styrax benzoin (benzoin)	*Mentha piperita* (peppermint)	**1.00 (±0.00)**	0.63	2.00 (±0.00)	1.00	2.00 (±0.00)	**0.50**	**1.00 (±0.00)**	0.67	**1.00 (±0.00)**	**0.25**	2.00 (±0.00)	1.00	**1.00 (±0.00)**	0.75	4.00 (±0.00)	2.00	**0.13 (±0.00)**	**0.21**
	Rosa damascena (rose)	2.00 (±0.00)	0.75	**1.00 (±0.00)**	**0.50**	**1.00 (±0.00)**	**0.38**	**0.50 (±0.00)**	2.17	1.50 (±0.71)	0.56	**0.50 (±0.00)**	**0.38**	2.00 (±0.00)	1.00	**1.00 (±0.00)**	**0.50**	**0.50 (±0.00)**	1.17
Syzygium caryophyllata (clove)	*Juniperus virginiana* (cedarwood)	**0.50 (±0.00)**	0.63	**0.50 (±0.00)**	**0.50**	**1.00 (±0.00)**	0.75	**0.19 (±0.00)**	0.55	**1.00 (±0.00)**	**0.50**	**0.38 (±0.18)**	1.13	**1.00 (±0.00)**	**0.50**	2.00 (±0.00)	2.00	**0.13 (±0.00)**	1.13
	Melaleuca alternifolia (tea tree)	**1.00 (±0.00)**	**0.38**	**1.00 (±0.00)**	0.83	**1.00 (±0.00)**	0.75	**0.50 (±0.00)**	**0.29**	**1.00 (±0.00)**	**0.38**	**0.50 (±0.00)**	1.17	**1.00 (±0.00)**	**0.38**	2.00 (±0.00)	1.50	**0.06 (±0.00)**	0.54
	Thymus vulgaris (thyme)	**1.00 (±0.00)**	**0.38**	**0.50 (±0.00)**	**0.50**	**0.50 (±0.00)**	**0.50**	**1.00 (±0.00)**	1.25	4.00 (±0.00)	1.50	**0.50 (±0.00)**	1.50	**0.50 (±0.00)**	**0.25**	2.00 (±0.00)	2.00	**0.13 (±0.00)**	1.33

Table 1. *Cont.*

Essential Oil Combinations (Common Name in Brackets)		Mean MIC Value (mg/mL) ($n = 3$) and ΣFIC																	
		Staphylococcus aureus		*Streptococcus agalactiae*		*Streptococcus pneumoniae*		*Streptococcus pyogenes*		*Mycobacterium smegmatis*		*Haemophilus influenzae*		*Klebsiella pneumoniae*		*Moraxella catarrhalis*		*Cryptococcus neoformans*	
		(ATCC 25924)		(ATCC 55618)		(ATCC 49619)		(ATCC 12344)		(ATCC 19420)		(ATCC 19418)		(ATCC 13883)		(ATCC 23246)		(ATCC 14116)	
Essential Oil 1	**Essential Oil 2**	**MIC ***	**ΣFIC ****	**MIC ***	**ΣFIC ****	**MIC ***	**ΣFIC ****	**MIC ***	**ΣFIC ****	**MIC ***	**ΣFIC ****	**MIC ***	**ΣFIC ****	**MIC ***	**ΣFIC ****	**MIC ***	**ΣFIC ****	**MIC ***	**ΣFIC ****
Positive control (ciprofloxacin)		0.50×10^{-3}		0.50×10^{-3}		0.50×10^{-3}		0.50×10^{-3}		0.50×10^{-3}		0.25×10^{-3}		1.00×10^{-3}		0.50×10^{-3}		n.a.	
Positive control (amphotericin b)		n.a.		n.a.		n.a.		n.a.		n.a.		n.a.		n.a.		n.a.		0.50×10^{-3}	
Negative control (acetone in water)		>8.00		>8.00		>8.00		>8.00		>8.00		>8.00		>8.00		>8.00		>8.00	

MIC * denotes noteworthy antimicrobial effect (MIC less than or equal to 1 mg/mL); ΣFIC ** in bold and italics denotes synergistic antimicrobial effect (ΣFIC less than or equal to 0.50); while ΣFIC in italics only denotes additive antimicrobial effect (ΣFIC greater than 0.50 and less than or equal to 1.00).

The pathogen most sensitive to the effects of the essential oil combinations was *Cryptococcus neoformans* (average MIC of 0. 49 mg/mL). The antimicrobial effect of essential oils against fungal pathogens has been well studied against the test micro-organisms investigated here-in [34,35]. The genus of *Cryptococcus* is an important cause of opportunistic fungal infection in severely immunocompromised patients with the primary sites of infection including the lungs [36]. The lowest MIC value determined for the combination of essential oils against *C. neoformans* was 0.06 mg/mL for 24 combinations including the combination of *Citrus limon* (lemon) and *Santalum austrocaledonicum* (sandalwood), which were shown to have broad-spectrum activity. The essential oils of *C. limon* and *S. austrocaledonicum* have demonstrated antimicrobial activity with marked antifungal effects [33,37–39].

The respiratory tract pathogens most commonly neglected in research include *Haemophilus influenzae* and the *Mycobacterium* and *Streptococcal* species [15]. Of these neglected pathogens, *M. smegmatis* demonstrated the greatest sensitivity to essential oils with 249 combinations proving synergistic and no antagonism noted (supplementary data, Table S1). The use of essential oils against tuberculosis has been commonly utilized with practices ranging from the inhalation of essential oils [40], to the application of essential oils within a diffuser to reduce bacterial loads at patient's bedsides [41,42]. A commonly used essential oil combination for the reduction of *Mycobacterium* air content include a mixture of *Melaleuca quinquenervia* (niaouli), *Eucalyptus smithii* (eucalyptus), *Myrtus communis* (myrtle), *Abies balsamea* (fir balsam), *Melaleuca alternifolia* (tea tree), *Pelargonium asperum* (geranium) and *Mentha piperita* (peppermint) essential oils [42]. Of these essential oils studied in 1:1 ratios, the ΣFIC values ranged from 0.42 to 0.50, further supporting their synergistic potential for use in combination. Essential oils have demonstrated moderate antimicrobial effects against *H. influenzae* and the *Streptococcal* pathogens commonly associated with respiratory infections [43–49]. These findings were supported in the current study.

Several additive and synergistic combinations could be observed with 57.1% of combinations demonstrating these effects. Antagonism was noted in only 5.4% of combinations. The combination of *Coriandrum sativum* (coriander) and *Cinnamonum zeylanicum* (cinnamon) showed the broadest spectrum of antimicrobial effect with noteworthy effect against eight of the nine pathogens studied with synergistic or additive effects against five of the nine pathogens. These two essential oils have been used since antiquity for their antimicrobial effects [50,51]. An additional two combinations, namely *C. zeylanicum* in combination with *Zingiber officinale* (ginger) and *Citrus bergamia* (bergamot) in combination with *Rosmarinus officinalis* (rosemary) have shown the greatest number of synergistic interactions with synergy identified against six of the nine pathogens investigated.

2.2. Brine-Shrimp Lethality Assay (BSLA)

All essential oils and their 1:1 combinations were screened at 1 mg/mL, where toxicity was considered where the essential oils induced mortality at a percentage greater than 50% [52]. Of the combinations investigated (Supplementary Table S2), the toxicity results of the single oils (a) and combinations (b) demonstrating the least toxicity are summarized in Figure 2.

Approximately 31.0% of the combinations studied demonstrated non-toxic effects against the brine shrimp. From these results, it appears that the essential oils show the potential to quench toxicity of individual essential oils when used in combination. Selected combinations (Table 2) which were based on the good antimicrobial activity indicated that although the combinations remain non-toxic, 76.0% of essential oils increase in toxicity when combined. The essential oil toxicity after 48 h when studied individually increased from 20.4% to 42.5% once combined. The combination of *Syzygium caryophyllata* (clove) with *C. zeylanicum*, or with *Thymus vulgaris* (thyme) showed the greatest synergistic (non-toxic effects) against brine shrimp with an ΣFIC of 0.03; followed by the combination of *S. caryophyllata* with *Cymbopogon citratus* (lemongrass) with an ΣFIC of 0.05.

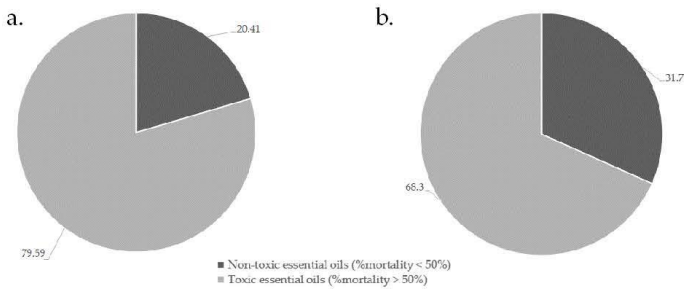


Figure 2. Summary of essential oil toxic effects alone (**a**) and when in combination (**b**) against brine shrimp.

Table 2. The mean percentage brine-shrimp viability after 48 h (n = 3) with standard deviation in brackets and ΣFIC values of the essential oil combinations investigated.

Essential Oil Combinations		Individual Brine-Shrimp Mortality (%) *		Combined Brine-Shrimp Mortality (%) *	ΣFIC **
Essential Oil 1	**Essential Oil 2**	**Essential Oil 1**	**Essential Oil 2**		
Amyris balsamifera (amyris)	*Styrax benzoin* (benzoin)	**1.4 (±0.89)**	**2.47 (±1.08)**	**5.65 (±4.78)**	3.16
Citrus bergamia (bergamot)	*Cupressus sempervirens* (cypress)	**1.22 (±1.55)**	**1.02 (±2.96)**	**0.65 (±1.13)**	*0.59*
Cupressus sempervirens (cypress)	*Hyssopus officinalis* (hyssop)	**1.02 (±2.96)**	**6.65 (±4.54)**	**8.80 (±4.98)**	4.99
	Lavandula angustifolia (lavender)		**9.59 (±3.23)**	**39.18 (±3.98)**	21.29
	Melaleuca alternifolia (tea tree)		**1.07 (±0.56)**	**8.87 (±1.71)**	8.48
	Salvia sclarea (clary sage)		**0.20 (±0.35)**	**11.81 (±5.88)**	34.67
Elettaria cardamonum (cardamom)	*Coriandrum sativum* (coriander)	**0.00 (±0.00)**	**11.95 (±2.96)**	**35.07 (±9.30)**	1754.74
Ferula galbaniflua (galbanum)	*Zingiber officinale* (ginger)	**15.77 (±0.09)**	**0.19 (±0.33)**	**28.08**	74.18
Helichrysum italicum (immortelle)	*Lavandula angustifolia* (lavender)	**9.50 (±4.54)**	**9.59 (±3.23)**	**13.45 (±11.74)**	1.41
	Lavandula spica (lavender spike)		**1.01 (±0.47)**	**26.38 (±5.63)**	14.46
Hyssopus officinalis (hyssop)	*Rosmarinus officinalis* (rosemary)	**6.65 (±4.54)**	**0.71 (±0.73)**	**13.07 (±1.68)**	10.23
Laurus nobilis (bay)	*Eucalyptus globulus* (eucalyptus)	**3.04 (±1.50)**	**4.21 (±1.09)**	**17.86 (±1.73)**	5.06
Lavandula angustifolia (lavender)	*Citrus aurantifolia* (lime)	**9.59 (±3.23)**	**0.36 (±0.62)**	**33.87 (±2.42)**	48.51
Myrtus communis (myrtle)	*Melaleuca alternifolia* (tea tree)	**0.00 (±0.00)**	**1.07 (±0.56)**	**28.33 (±4.76)**	1429.46
Ocimum basilicum (basil)		**13.04 (±6.25)**	**1.07 (±0.56)**	**39.69 (±2.34)**	19.99
Origanum marjorana (marjoram)		**0.34 (±6.25)**	**1.07 (±0.56)**	**6.03 (±1.85)**	11.76
Origanum vulgare (origanum)		99.67 (±0.57)	**1.07 (±0.56)**	**7.75 (±2.49)**	3.65

Table 2. *Cont.*

Essential Oil Combinations		Individual Brine-Shrimp Mortality (%) *		Combined Brine-Shrimp Mortality (%) *	ΣFIC **
Essential Oil 1	**Essential Oil 2**	**Essential Oil 1**	**Essential Oil 2**		
Salvia sclarea (clary sage)	*Citrus aurantifolia* (lime)	**0.20 (±0.35)**	**0.36 (±0.62)**	56.47 (±5.09)	80.06
	Citrus bergamia (bergamot)		**1.22 (±1.55)**	**15.76 (±5.97)**	128.15
	Lavandula burnati (lavandin)		**0.16 (±0.26)**	**12.64 (±3.46)**	279.78
Styrax benzoin (benzoin)	*Mentha piperita* (peppermint)	**2.47 (±1.08)**	**6.98 (±1.07)**	**3.31 (±1.09)**	12.28
	Rosa damascena (rose)		**97.32 (±2.34)**	**3.11 (±1.76)**	2.92
Syzygium caryophyllata (clove)	*Cinnamonum zeylanicum* (cinnamon)	99.57 (±0.74)	**98.49 (±0.52)**	**6.58 (±1.57)**	***0.03***
	Cymbopogon citratus (lemongrass)		**100 (±0.00)**	**2.30 (±1.24)**	***0.05***
	Juniperus virginiana (cederwood)		**0.57 (±0.23)**	**3.06 (±1.05)**	3.91
	Thymus vulgaris (thyme)		**97.76 (±2.56)**	**25.16 (±4.52)**	***0.03***
Positive control (Potassium dichromate)		99.95 (±0.03)			
Negative control (Sea water)		0.00 (±0.00)			
Solvent control (DMSO)		0.00 (±0.00)			

Brine-shrimp viability (%) * in bold denotes non-toxic effect (% mortality less than 50%); ΣFIC ** in bold denotes synergistic effect (ΣFIC less than or equal to 0.50) and ΣFIC in italics denotes additive effect (ΣFIC greater than 0.50 and less than or equal to 1.00).

While the BSLA serves as an excellent tool to identify toxicity, a more sensitive distinction of activity is the testing against cell lines [53]. Hence, the BSLA was used to identify combinations of interest, which were then followed by the MTT assay using RAW 264.7 macrophages and A549 lung cell lines to substantiate the results of the BSLA as the latter study considers the metabolic activity of the cells.

2.3. Inhibition of LPS-Induced Macrophage Activation

From the results derived in the combined antimicrobial assays, and the BSLA, 24 essential oil combinations were found to be non-toxic and have broad-spectrum antimicrobial activity, and were therefore selected for further anti-inflammatory studies. The anti-inflammatory effect was studied by measuring the production of a single inflammatory marker, nitric oxide (NO). As inflammation is a very complex process, this assay was applied to determine the preliminary potential of essential oils for anti-inflammatory effects. Additional toxicity studies against lung tissue were undertaken to further identify the potentially toxic effects of essential oils commonly inhaled for respiratory conditions.

Statistical analysis was undertaken using StatSoft Inc. (Tulsa, OK, USA) (2004) STATISTICA (Data Analysis Software System), Version 7 software. An area under the curve (AUC) and Receiver Operator Characteristic (ROC) study was performed to determine the optimum cut-off point for the production of NO. This was created taking into consideration cell cytotoxicity. The cut-off for essential oils demonstrating anti-inflammatory activity, relevant to cell cytotoxicity, was therefore determined as 2.97 μM nitrite production. The majority of essential oil combinations studied (62.5%), demonstrated an anti-inflammatory effect when compared to LPS, with anti-inflammatory activity demonstrated as 1.98 to 2.97 μM nitrite production compared to the control, LPS at 4.90 μM. Figure 3 summarizes the percentage of essential oils with anti-inflammatory effect when tested independently (a) and when in combination (b).

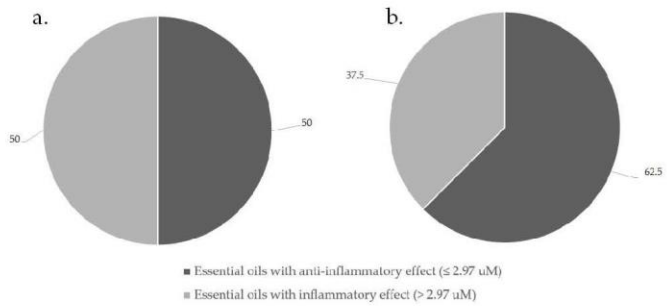


Figure 3. Summary of essential oil anti-inflammatory effects alone (**a**) and when in combination (**b**).

From these results, essential oils showed an increased potential for anti-inflammatory effects when used in combination. Table 3 represents the results for the essential oils investigated individually and the 24 selected combinations (1:1) for anti-inflammatory effect.

Table 3. The mean percentage NO production of RAW 264.7 macrophages ($n = 3$) with standard deviation in brackets and ΣFIC values after treatment with essential oil combinations.

Essential Oil Combination		Individual Essential Oil Nitrite Production (μM)		Combined Essential Oils Nitrite Production (μM) *	ΣFIC *** % NO Production
Essential Oil 1	**Essential Oil 2**	**Essential oil 1**	**Essential oil 2**		
Amyris balsamifera (amyris)	*Styrax benzoin* (benzoin)	1.95 (±0.02)	2.45 (±0.07)	*2.22 (±0.03)*	1.02
Ocimum basilicum (basil)	*Melaleuca alternifolia* (tea tree)	2.91 (±0.19)	4.06 (±0.23)	3.83 (±0.10)	1.13
Laurus nobilis (bay)	*Eucalyptus globulus* (eucalyptus)	5.43 (±0.38)	4.57 (±0.19)	4.04 (±0.05)	*0.81*
Styrax benzoin (benzoin)	*Mentha piperita* (peppermint)	2.45 (±0.07)	6.38 (±0.44)	3.75 (±0.05)	1.06
	Rosa damascene (rose)	2.45 (±0.07)	4.71 (±0.40)	***2.08 (±0.01)***	*0.65*
Citrus bergamia (bergamot)	*Cupressus sempervirens* (cypress)	2.17 (±0.10)	2.02 (±0.10)	***2.05 (±0.00)***	*0.98*
Elettaria cardamonum (cardamom)	*Coriandrum sativum* (coriander)	2.12 (±0.06)	1.93 (±0.02)	***2.01 (±0.03)***	*0.99*
Salvia sclarea (clary sage)	*Citrus bergamia* (bergamot)	2.02 (±0.02)	2.17 (±0.10)	***2.07 (±0.03)***	*0.99*
	Lavandula burnati (lavandin)	2.02 (±0.02)	3.61 (±0.05)	4.30 (±0.05)	1.66
	Citrus aurantifolia (lime)	2.02 (±0.02)	2.00 (±0.02)	***2.02 (±0.05)***	1.00
Syzygium caryophyllata (clove)	*Juniperus virginiana* (cedarwood)	1.95 (±0.02)	3.38 (±0.02)	***2.01 (±0.03)***	*0.81*
	Melaleuca alternifolia (tea tree)	1.95 (±0.02)	4.06 (±0.23)	***2.07 (±0.03)***	*0.79*
Cupressus sempervirens (cypress)	*Salvia sclarea* (clary sage)	2.02 (±0.10)	2.02 (±0.02)	***2.00 (±0.05)***	*0.99*
	Hyssopus officinalis (hyssop)	2.02 (±0.10)	3.59 (±0.07)	***1.98 (±0.02)***	*0.77*
	Lavandula angustifolia (lavender)	2.02 (±0.10)	2.99 (±0.38)	***2.00 (±0.03)***	*0.83*
	Melaleuca alternifolia (tea tree)	2.02 (±0.10)	4.06 (±0.23)	3.49 (±0.02)	1.30

Table 3. *Cont.*

Essential Oil Combination		Individual Essential Oil Nitrite Production (μM)		Combined Essential Oils Nitrite Production (μM) *	ΣFIC *** % NO Production
Essential Oil 1	**Essential Oil 2**	**Essential oil 1**	**Essential oil 2**		
Ferula galbaniflua (galbanum)	*Zingiber officinale* (ginger)	1.99 (±0.03)	2.85 (±0.04)	***2.34 (±0.03)***	1.00
Hyssopus officinalis (hyssop)	*Rosmarinus officinalis* (rosemary)	3.59 (±0.07)	4.73 (±0.18)	3.54 (±0.03)	*0.87*
Helichrysum italicum (immortelle)	*Lavandula angustifolia* (lavender)	2.06 (±0.02)	2.99 (±0.04)	***2.04 (±0.02)***	*0.84*
	Lavandula spica (lavender spike)	2.06 (±0.02)	4.68 (±0.17)	3.44 (±0.18)	1.20
Lavandula angustifolia (lavender)	*Citrus aurantifolia* (lime)	2.99 (±0.04)	2.00 (±0.02)	***2.97 (±0.05)***	1.24
Origanum marjorana (marjoram)	*Melaleuca alternifolia* (tea tree)	4.81 (±0.11)	4.06 (±0.23)	3.05 (±0.14)	*0.69*
Myrtus communis (myrtle)		3.07 (±0.13)	4.06 (±0.23)	***2.97 (±0.01)***	*0.85*
Origanum vulgare (origanum)		4.88 (±0.47)	4.06 (±0.23)	3.87 (±0.18)	*0.87*
Medium only			1.77 (±4.52)		
Positive control (LPS + Aminoguanidine)			2.58 (±2.39)		
Negative control (LPS + medium)			4.90 (±9.21)		

Combined essential oil NO production (%) * bold and in italics denotes anti-inflammatory effect (μM less than 2.97); ΣFIC *** in bold denotes synergistic effect (ΣFIC less than or equal to 0.50); ΣFIC in italics denotes additive effect (ΣFIC greater than 0.50 but less than or equal to 1.00).

The essential oil combination with the greatest anti-inflammatory effect was *Cupressus sempervirens* (cypress) with *Hyssopus officinalis* (hyssop) having a nitrite production as low as 1.98 μM. Previous studies have shown the anti-inflammatory potential of these essential oils [54], however, little is known of the combined anti-inflammatory effects of these essential oils identified in this study [15]. To confirm the absence of cytotoxicity caused by exposure of the RAW macrophage cells to these essential oil combinations, leading to a reduced NO production, an MTT assay was run concurrently. The results of the MTT assay are provided in Table 4.

Table 4. The mean percentage cell viability of RAW 264.7 macrophages (n = 3) with standard deviation in brackets and ΣFIC values after treatment with essential oil combinations.

Essential Oil Combination		Cell Viability against Individual Essential Oils (%)		Cell Viability against Combined Essential Oils (%) **	ΣFIC *** % Cell Viability
Essential Oil 1	**Essential Oil 2**	**Essential Oil 1**	**Essential Oil 2**		
Amyris balsamifera (amyris)	*Styrax benzoin* (benzoin)	8.11 (±4.52)	47.98 (±0.08)	7.90 (±4.05)	*0.57*
Ocimum basilicum (basil)	*Melaleuca alternifolia* (tea tree)	50.63 (±2.39)	66.15 (±2.07)	***66.80 (±3.90)***	1.16
Laurus nobilis (bay)	*Eucalyptus globulus* (eucalyptus)	58.49 (±9.20)	67.01 (±0.23)	***74.91 (±0.15)***	1.2
Styrax benzoin (benzoin)	*Mentha piperita* (peppermint)	47.98 (±0.08)	61.83 (±3.25)	58.45 (±0.09)	1.08
	Rosa damascene (rose)		60.04 (±3.54)	36.90 (±1.75)	*0.69*
Citrus bergamia (bergamot)	*Cupressus sempervirens* (cypress)	33.40 (±4.98)	7.98 (±0.33)	8.68 (±2.65)	*0.67*
Elettaria cardamonum (cardamom)	*Coriandrum sativum* (coriander)	8.11 (±3.77)	9.12 (±5.27)	8.11 (±2.65)	*0.94*

Table 4. *Cont.*

Essential Oil Combination		Cell Viability against Individual Essential Oils (%)		Cell Viability against Combined Essential Oils (%) **	ΣFIC *** % Cell Viability
Essential Oil 1	**Essential Oil 2**	**Essential Oil 1**	**Essential Oil 2**		
Salvia sclarea (clary sage)	*Citrus bergamia* (bergamot)	14.99 (±0.15)	33.40 (±4.98)	8.07 (±0.88)	**0.39**
	Lavandula burnati (lavandin)		71.04 (±0.15)	55.64 (±0.69)	2.25
	Citrus aurantifolia (lime)		12.3 (±4.82)	8.15 (±0.15)	*0.60*
Syzygium caryophyllata (clove)	*Juniperus virginiana* (cederwood)	23.83 (±3.70)	57.68 (±3.99)	7.98 (±0.09)	**0.24**
	Melaleuca alternifolia (tea tree)		66.15 (±2.07)	51.65 (±0.23)	1.47
Cupressus sempervirens (cypress)	*Salvia sclarea* (clary sage)	7.98 (±0.33)	14.99 (±0.15)	9.94 (±0.23)	*0.95*
	Hyssopus officinalis (hyssop)		63.14 (±0.09)	12.18 (±0.13)	*0.86*
	Lavandula angustifolia (lavender)		71.53 (±3.44)	9.08 (±0.23)	*0.63*
	Melaleuca alternifolia (tea tree)		66.15 (±2.07)	***83.83 (±8.08)***	5.88
Ferula galbaniflua (galbanum)	*Zingiber officinale* (ginger)	8.07 (±5.13)	8.11 (±1.63)	8.07 (±1.74)	*1.00*
Hyssopus officinalis (hyssop)	*Rosmarinus officinalis* (rosemary)	63.14 (±0.09)	77.64 (±0.84)	***79.59 (±3.12)***	1.14
Helichrysum italicum (immortelle)	*Lavandula angustifolia* (lavender)	7.94 (±0.09)	71.53 (±3.44)	8.27 (±0.48)	*0.58*
	Lavandula spica (lavender spike)		68.92 (±0.08)	***72.06 (±1.93)***	5.06
Lavandula angustifolia (lavender)	*Citrus aurantifolia* (lime)	71.53 (±3.44)	12.3 (±4.82)	***68.84 (±0.09)***	3.28
Origanum marjorana (marjoram)	*Melaleuca alternifolia* (tea tree)	57.84 (±0.28)	66.15 (±2.07)	***90.14 (±0.16)***	1.46
Myrtus communis (myrtle)		59.43 (±4.00)		***78.00 (±4.67)***	1.25
Origanum vulgare (origanum)		68.84 (±3.69)		***86.31 (±0.58)***	1.28
LPS		109.31 (±0.04)			
Positive control (Adenosine Guanine)		119.51 (±0.23)			
Negative control (LPS + medium)		100.00 (±0.18)			

Combined essential oil cell viability (%) ** bold and italics denotes non-toxic effect (%cell viability greater than 50%); ΣFIC *** in bold denotes synergistic effect (ΣFIC less than or equal to 0.50); ΣFIC in italics denotes additive effect (ΣFIC greater than 0.50 but less than or equal to 1.00).

The results of the MTT assay indicate that cytotoxicity is evident in 50% of the essential oil combinations towards the RAW 264.7 cells, providing reduced confidence in the anti-inflammatory results associated to these combinations as these may be meaningfully confounded by cell death. Of the combinations studied, three combinations demonstrated cell viability above 80% and NO production less than 4.00 µM, namely *Origanum marjorana* (marjoram) with *M. alternifolia* (3.05 uM, 90.14%), *Origanum vulgare* (origanum) with *M. alternifolia* (3.87 uM, 86.31%), and C. *sempervirens* with *M. alternifolia* (3.49, 83.83%).

Numerous studies support the anti-inflammatory activity of *M. alternifolia* essential oil both *in vitro* and *in vivo* [55–58]. Studies have shown the potent effects of *M. alternifolia* essential oil in inhibiting the production of the inflammatory mediators tumour necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), IL-10, and that of prostaglandin E2 [58].

Previous studies support the anti-inflammatory effect of *Origanum* essential oils [59–61]. Research on the anti-inflammatory effect of the essential oils *O. marjoram* and *O. vulgare* determined marked anti-inflammatory effect of these oils against TNFα, IL-1β, IL-6, IL-10 and inhibition of cyclooxygenase-2 [59,61]. These findings thus support those provided in literature of the essential oils *M. alternifolia*, *O. marjoram* and *O. vulgare* to produce anti-inflammatory effects and further demonstrate increased efficacy in combination.

2.4. Cytotoxic Effects of Essential Oils against A549 Lung Cancer Cells

To evaluate the cytotoxic effects of the essential oils and essential oil combinations against cells of the lung, the A549 cell line was exposed over a 48 h period and examined by the MTT assay. The results for 24 essential oil combinations are provided in Table 5.

Table 5. The mean percentage cell viability of A549 lung cancer cell line ($n = 3$) with standard deviation in brackets and ΣFIC values after treatment with essential oil combinations.

Essential Oil Combination		A549 Cell Viability * Following Individual Essential Oils (%)		A549 Cell Viability * Following Combined Essential Oils (%)	ΣFIC % Cell Viability **
Essential Oil 1	**Essential Oil 2**	**Essential Oil 1**	**Essential Oil 2**		
Amyris balsamifera (amyris)	*Styrax benzoin* (benzoin)	**94.78 (±0.08)**	**91.25 (±11.08)**	**87.95 (±0.09)**	*0.95*
Ocimum basilicum (basil)	*Melaleuca alternifolia* (tea tree)	**87.16 (±4.98)**	**86.01 (±3.90)**	**79.62 (±1.75)**	*0.92*
Laurus nobilis (bay)	*Eucalyptus globulus* (eucalyptus)	**89.55 (±2.89)**	**99.63 (±4.69)**	**96.14 (±2.65)**	1.02
Styrax benzoin (benzoin)	*Mentha piperita* (peppermint)	**91.25 (±11.08)**	**94.95 (±3.54)**	**95.22 (±2.65)**	1.02
	Rosa damascena (rose)		**87.84 (±5.17)**	**88.38 (±0.88)**	*0.99*
Citrus bergamia (bergamot)	*Cupressus sempervirens* (cypress)	**96.41 (±8.23)**	**100.00 (±0.13)**	**93.88 (±0.69)**	*0.96*
Elettaria cardamonum (cardamom)	*Coriandrum sativum* (coriander)	**84.15 (±0.08)**	**97.36 (±0.23)**	**89.47 (±0.15)**	*0.99*
Salvia sclarea (clary sage)	*Citrus bergamia* (bergamot)	**99.72 (±0.33)**	**96.41 (±8.23)**	**94.11 (±0.09)**	*0.96*
	Cupressus sempervirens (cypress)		**100.00 (±0.13)**	**98.28 (±0.23)**	*0.98*
	Lavandula burnati (lavandin)		**98.88 (±0.74)**	**87.41 (±2.30)**	*0.88*
	Citrus aurantifolia (lime)		**100.00 (±4.00)**	**82.00 (±0.13)**	*0.82*
Syzygium caryophyllata (clove)	*Juniperus virginiana* (cedarwood)	**99.36 (±10.45)**	**95.86 (±3.70)**	**99.55 (±0.23)**	1.02
	Melaleuca alternifolia (tea tree)		**86.01 (±3.90)**	**81.75 (±8.08)**	*0.89*
Cupressus sempervirens (cypress)	*Salvia sclarea* (clary sage)	**100.00 (±0.13)**	**99.72 (±0.33)**	**96.66 (±1.74)**	*0.97*
	Hyssopus officinalis (hyssop)		**93.14 (±3.44)**	**98.00 (±3.12)**	1.02
	Lavandula angustifolia (lavender)		**99.21 (±6.20)**	**98.41 (±0.48)**	*0.99*
	Melaleuca alternifolia (tea tree)		**86.01 (±3.90)**	**87.23 (±1.93)**	*0.94*
Ferula galbaniflua (galbanum)	*Zingiber officinale* (ginger)	**100.00 (±0.00)**	**95.85 (±0.15)**	**99.42 (±0.09)**	1.02

Table 5. *Cont.*

Essential Oil Combination		A549 Cell Viability * Following Individual Essential Oils (%)		A549 Cell Viability * Following Combined Essential Oils (%)	ΣFIC % Cell Viability **
Essential Oil 1	**Essential Oil 2**	**Essential Oil 1**	**Essential Oil 2**		
Hyssopus officinalis (hyssop)	*Rosmarinus officinalis* (rosemary)	**93.14 (±3.44)**	**89.15 (±4.68)**	**96.23 (±0.16)**	1.06
Helichrysum italicum (immortelle)	*Lavandula angustifolia* (lavender)	**99.05 (±3.44)**	**99.21 (±6.20)**	**98.18 (±4.67)**	*0.99*
	Lavandula spica (lavender spike)		**99.31 (±4.82)**	**97.58 (±0.58)**	*0.98*
Lavandula angustifolia (lavender)	*Citrus aurantifolia* (lime)	**99.21 (±6.20)**	**100.00 (±4.00)**	**80.38 (±2.90)**	*0.81*
Origanum marjorana (marjoram)	*Melaleuca alternifolia* (tea tree)	**96.52 (±3.79)**	**86.01 (±3.90)**	**89.88 (±3.11)**	*0.99*
Myrtus communis (myrtle)		**98.24 (±3.69)**		**80.73 (±9.14)**	*0.88*
Origanum vulgare (origanum)		**95.03 (±3.43)**		**91.17 (±0.86)**	1.01
Positive control (Melphalan)		66.37 (±0.26)			
Negative control (Medium control)		92.30 (±0.27)			

A549 cell viability (%) * denotes non-toxic effects (% cell viability greater than 50%); ΣFIC ** in bold denotes synergistic effect (ΣFIC less than or equal to 0.50); ΣFIC in italics denotes additive effect (ΣFIC greater than 0.50 but less than or equal to 1.00).

The essential oils retained cell viability between 84.2% and 100.0%, while the combinations showed A549 cell viability between 80.4% and 99.6%. Consequently, all essential oils and their combinations showed non-toxic effects against the lung A549 cell lines. These findings further support those determined in the BSLA and provide an initial indication for the safe therapeutic potential of essential oils when used in the respiratory tract. In the majority (75.0%) of the combinations studies, additive effects were demonstrated when essential oils were tested against A549 cell lines. Among the tested essential oil combinations, *Lavandula angustifolia* (lavender) and *Citrus aurantifolia* (lime) exhibited the lowest (i.e., best effect in combination) additive effects (ΣFIC = 0.81) to the A549 cells compared to the other combinations.

3. Materials and Methods

The methods undertaken in this study aim to determine the best essential oil combination for use in the respiratory tract. All studies were undertaken in triplicate on consecutive days. Varying methods were applied to identify the most suitable combination of essential oil from the initial 369 combinations studied. An overview of the study design is provided in Figure 4.

3.1. Essential Oil Combination Selection, Procurement and Chemical Characterization

A selection of 49 essential oils were obtained from international fragrance companies Robertet© (Grasse, France), Burgess and Finch (Cape Town, South Africa), PranaMonde (Belgium) and Escentia Oils (Gauteng, South Africa). The essential oil combinations were selected based on the frequency of citation in the aroma-therapeutic literature available to the layman, with specified recommendation in treating respiratory tract infections [62–68]. The chemical analysis for all oils in this study has previously been characterized using gas chromatography coupled with a mass spectrometer (GC/MS) and is reported in our earlier studies [33].

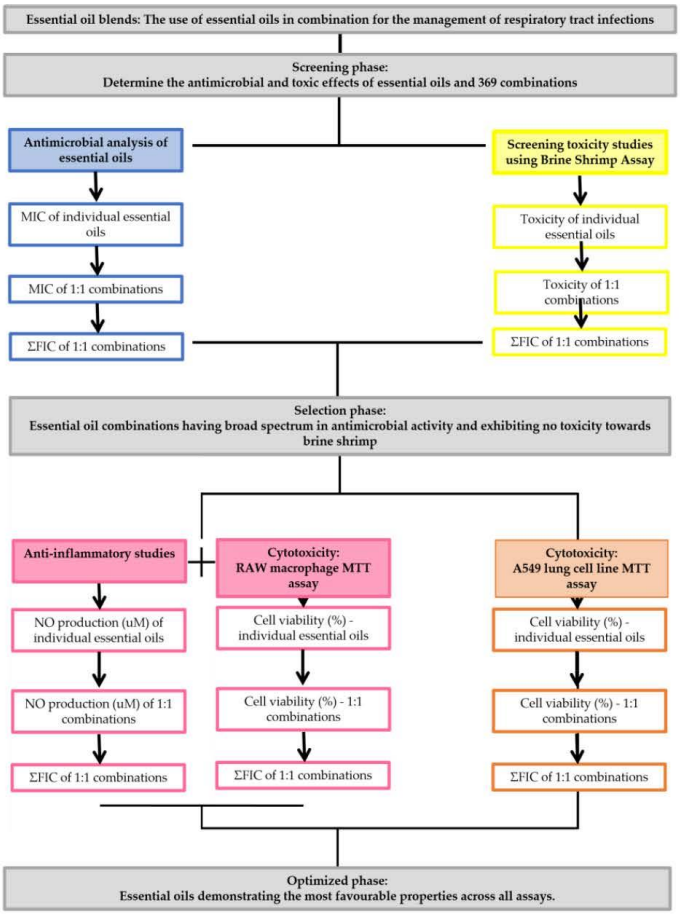


Figure 4. Summary of study workflow.

3.2. *Antimicrobial Analysis*

Microbial cultures were selected based on their relevance to respiratory infections and included the Gram-positive strains *Staphylococcus aureus* (ATCC 25924), *Streptococcus agalactiae* (ATCC 55618), *Streptococcus pneumoniae* (ATCC 49619) and *Streptococcus pyogenes* (ATCC 12344) and the Gram-negative strains *Haemophilus influenzae* (ATCC 19418), *Klebsiella pneumoniae* (ATCC 13883) and *Moraxella catarrhalis* (ATCC 23246). The non-pathogenic *Mycobacterium* strain *M. smegmatis* (ATCC 19420) and yeast strain *Cryptococcus neoformans* (ATCC 14116) were also selected for analysis. All cultures were prepared as per Leigh-de Rapper et al. [33]. A waiver for the use of these micro-organisms was granted by the University of the Witwatersrand Human Research Ethics Committee (Reference W-CJ-160720-2).

The broth microdilution method described by de Rapper et al. [18] was used to quantify the antimicrobial inhibitory activity of the selected essential oils. The antimicrobial activities of the essential oils independently have been previously investigated [33]. The combinations of essential oils were undertaken at 1:1 ratios. The micro-titre plates were prepared aseptically [33]; however, a stock concentration of each essential oil (32 mg/mL

in acetone) in the 1:1 combination was added to the first row at a volume of 50 µL per oil. The MIC values were recorded and the fractional inhibitory concentration index (ΣFIC) was calculated according to the review by van Vuuren and Viljoen [13] using the following equations;

$$\text{FIC (i)} = \frac{\text{MIC of (a*) combined with (b*)}}{\text{MIC of (a) independently}}$$

$$\text{FIC (ii)} = \frac{\text{MIC of (b) combined with (a)}}{\text{MIC of (b) independently}}$$

* where (a) is the MIC of the one oil in the combination and (b) is the MIC of the other.

From these values the FIC index was calculated as: ΣFIC = FIC (i) + FIC (ii). The ΣFIC for each oil combination was interpreted where an ΣFIC value of $\leq$0.5 is indicative of synergy, an ΣFIC value of >0.5–1.0 is additive, an ΣFIC of >1.0–$\leq$ 4.0 indicates indifference and an ΣFIC value of >4.0 indicates antagonism [13].

3.3. Brine-Shrimp Lethality Assay (BSLA)

The BSLA was used to quantify the toxic effects of the selected essential oils [69]. A volume of 400 µL salt water containing on average 40–60 live brine-shrimp was added to each well of a 48 well micro-titre plate. Thereafter, 400 µL of essential oil sample (essential oil or a combination of essential oils (1:1), all diluted in 1% dimethyl sulphoxide (DMSO)) was added to wells. All samples were tested for toxicity at a concentration of 1 mg/mL, since a concentration above 1 mg/mL not resulting in brine-shrimp death was considered non-toxic for the assay [52]. The negative control consisted of 32 g/L salt water, a solvent control of 1 mg/mL DMSO in water and the positive control consisted of 1.60 mg/mL potassium dichromate (Fluka). The micro-titre plates were observed under a light microscope (Olympus) (40 × magnification) immediately after sample addition (at time 0) for any dead brine-shrimp, which would be excluded from the percentage mortality calculations [70]. Dead brine-shrimp were then counted after 24 and 48 h. Thereafter, a lethal dose of 50 µL of glacial acetic acid (100% *v*/*v*; Saarchem, Maharashtra, India) was added to each well and a total dead brine-shrimp count undertaken. Samples providing a percentage mortality greater than 50% were considered toxic [52].

3.4. Anti-Inflammatory and MTT Cytotoxicity Assay

Forty-nine essential oils and twenty-four essential oil (1:1) combinations were selected for study based on the favorable results from the MIC assay and BSLA. The anti-inflammatory activity of these essential oil combinations were assessed by measuring the inhibition of nitric oxide (NO), an inflammatory mediator, production in LPS-activated RAW 264.7 macrophages. The control, lipopolysaccharides (LPS), is a potent inducer of nitric oxide synthase (iNOS) and concomitant NO producer, hence stimulating the signaling cascade that contributes to the inflammatory response in RAW 264.7 macrophage cells. Simultaneous evaluation of cell viability, using an MTT assay, was used to confirm the absence of cytotoxicity of these combinations. The essential oils were prepared in DMSO to a stock concentration of 10% and further diluted in culture medium to a concentration of 0.02%. RAW 264.7 mouse macrophage cells (Cellonex, South Africa) were cultured in RPMI complete medium, comprised of RPMI1640 supplemented with 10% fetal calf serum (GE Healthcare Life Sciences, (Logan, UT, USA)) in a humidified 5% CO_2 incubator at 37 °C. Cells were seeded into 96-well plates at a density of 1×10^5 cells per well and allowed to attach overnight. Spent culture medium was replaced with 50 µL of the samples (diluted in RPMI complete medium to 0.02%) or complete medium only (control and LPS control; Sigma-Aldrich, (St. Louise, MO, USA)) or aminoguanidine (AG; positive control; Sigma-Aldrich, (St. Louise, MO, USA)). To assess the anti-inflammatory activity, 50 µL of 1 µg/mL LPS in RPMI complete medium was added to all wells except controls, which received 50 µL medium. Final concentrations were 0.01% for essential oils, 500 ng/mL for

LPS and 100 µM for AG. Cells were incubated for a further 24 h. To quantify nitric oxide (NO) production, 50 µL of the spent culture medium was transferred to a new 96-well plate and 50 µL Griess reagent (Sigma-Aldrich, (St. Louise, MO, USA) was added. Samples were incubated at room temperature for 10 min before absorbance was measured at 540 nm and the results expressed relative to the appropriate untreated LPS control. A standard curve using sodium nitrite dissolved in culture medium was used to determine the concentration of NO in each sample. To confirm the absence of toxicity as a contributory factor, cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). This was done by replacing the remaining medium in each well with medium containing 0.5 mg/mL MTT and further incubation for 30 min at 37 °C. Thereafter, MTT was removed and 100 µL DMSO was added to each well to solubilize the formazan crystals. Absorbance was read at 540 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA).

3.5. Data Analysis

Data was analysed using StatSoft Inc. (Tulsa, OK, USA) (2004) STATISTICA (Data Analysis Software System), Version 7. Standard deviations were calculated to determine standard error of the mean. Further, logistic regression using an area under the curve (AUC) and Receiver Operator Characteristic (ROC) were applied to determine the optimum cut-off point for the production of NO by macrophages relevant to cell cytotoxicity.

4. Conclusions

This study elucidates the promising strategy of combining essential oils for use in the respiratory tract. This study is the first to report on the majority of these essential oil combinations against the pathogens of the respiratory tract as well as anti-inflammatory and cytotoxic effects on lung cell lines. Based on the findings of this study, five distinct combinations of essential oils have been determined as the most promising for use in the respiratory tract based on the antimicrobial, cytotoxic and anti-inflammatory effects. These combinations include; *C. sempervirens* in combination with *M. alternifolia*, *H. officinalis* in combination with *R. officinalis*, *O. marjorana* in combination with *M. alternifolia*, *M. communis* in combination with *M. alternifolia*, and *O. vulgare* in combination with *M. alternifolia*. Future studies are underway which examine the optimum ratios at which these essential oil combinations should be combined to provide a holistic blend that would be non-toxic and elicit the most favorable antimicrobial and anti-inflammatory activity.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/antibiotics10121517/s1, Table S1: The mean MIC ($n = 3$) and ΣFIC values of the essential oil combinations investigated against pathogens of the respiratory tract; Table S2: The mean percentage brine-shrimp viability after 48 h ($n = 3$) and ΣFIC values of the essential oil combinations investigated.

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Article 4 - Original Article on the optimization of essential oil combinations using isobolograms and SynergyFinder software

At the time of this thesis, this article had been accepted for publication. This article may not appear in the thesis due to copyright constraints but is available at the following DOI: 10.1055/a-1947-5680

CHAPTER 4:

ESSENTIAL OIL FORMULATION DESIGN



4. Introduction

The antimicrobial and therapeutic effects of essential oils have been widely accepted by the pharmaceutical industry. A large variety of inhalation essential oil preparations used to alleviate, manage, control and provide symptomatic relief of respiratory infections such as colds and coughs are available on the market. Table 4.1 provides a summary of the inhalable essential oil formulations available in South Africa.

Table 4.1: Comparative table of the inhalation essential oil formulations currently on the market in South Africa.

Product name and image	Ingredients	Indication as per manufacturer
Vicks inhaler nasal sticks 	125 mg Menthol; 50mg Camphor; 10 mg Siberian pine needle oil	The Vicks inhaler nasal stick is a decongestant agent used to provide symptomatic relief of the nasal passage. The essential oils work together and aid in breathing by lining the nasal passage and relieving nasal congestion caused by colds or allergies.
Vicks VapoRub 	4.8% Synthetic camphor; 1.2% Eucalyptus oil; 2.6% Menthol; Cedar leaf oil*; Nutmeg oil*; Petrolatum*; Thymol*; Turpentine oil*	Indicated for cough relief in persons two years and older. The vapours will last for eight hours.
Vicks VapoSteam 	27 mg/g Eucalyptus oil; 36 mg/g Peppermint oil; 10 mg/g Wintergreen; 2 mg/g Lavender oil	Vicks VapoSteam can either be used in vaporiser water, in an inhaler or in a hot water bowl to enable the inhalation of vapours. Indicated for cough relief and mucous reduction.
Vicks Vapo Inhaler 	415 mg Levomenthol; 415 mg Camphor	This formulation aids in decreasing nasal congestion and clearing the airways to help facilitate breathing in persons over 12 years of age.
Karvol 	103 mg Pumilio pine oil; 66 mg Terpineol; 35 mg Levomenthol; 9 mg *Pinus sylvestris* oil; 3 mg Thymol; 2 mg Chlorobutanol; Cinnamon oil*	Karvol is available as a gel that can be applied onto the skin and inhaled into the respiratory tract. Karvol is also available as inhalation capsules that can be added into a nebuliser. Karvol is indicated for the symptomatic management of nasal congestion, colds and bronchitis.

*percentage composition not disclosed; images taken from Google image search

4.1.Essential oil formulation development

Inhalation drug delivery systems aim to produce a system that is needle free and provides systemic delivery of small molecule containing drugs. A challenge to inhalation formulations is ensuring that particle sizes in the formulation are within the desired particle size range so that the active ingredient can be easily dispersed (Chaurasiya and Zhao, 2020). Particles in inhalation formulations are expressed in terms of the mass median aerodynamic diameter (MAAD). For particles to enter the respiratory tract by inertial impaction they need to be within 3 to 6 μm; while via sedimentation they need to be between 1 to 3 μm; and via diffusion the particles need be less than 1 μm. In order for an inhaled drug to reach the lower respiratory tract, the inhaled particle needs to be less than 5 μm (Jabbal et al., 2017). Additional limitations to essential oil formulation development include poor water solubility, toxicities and environmental instabilities of the essential oils due to heat, oxygen and moisture (Turek and Stintzing, 2013). Therefore, formulation techniques for delivery systems incorporating essential oils should be optimized to overcome these limitations while ensuring the pharmacological activity is retained (de Matos et al., 2019).

4.2. Drug delivery methodology in the formulation of essential oil containing products

Nanotechnology applications have great promise in pharmaceutical research and in the development of alternative drug delivery systems (Echeverría and Albuquerque, 2019). Nanotechnology involves the envelopment of nanoparticle drugs, or lipid bioactive molecules, into a homogenous or heterogenous matrix within a suitable nanocarrier (Ferreira and Nunes, 2019). Nanoencapsulation is advantageous as it improves bio-availability, cellular uptake and reduces associated toxicities. The efficacy, specificity and targeting capabilities of a therapeutic agent can be optimized while degradation by oxidation may also be prevented (Kumari et al., 2014). It also enables the encapsulation of poorly water-soluble molecules due the inherent increased solubilization capabilities (Wais et al., 2016). For an application in essential oils, these delivery systems have been proposed due to its effect in lowering volatility loss, increasing the stability, therapeutic efficacy, and aqueous solubility (Bilia et al., 2014). The preparation of an essential oil loaded nanocarrier is complex as there are various processes and steps involved that could affect the stability, bio-activity and organoleptic properties of the formulation. The choice of nanoencapsulation system should therefore be appropriately

selected for essential oil formulation. The various nanostructured systems are illustrated in Figure 4.1. A lipid base nanostructured system includes nanoemulsions, solid lipid nanoparticles, liposomes and self-emulsifying drug delivery systems. These systems are appropriate for pharmaceutical development of essential oils due to their ability to encapsulate lipophilic ingredients while maintaining product biodegradability and safety.

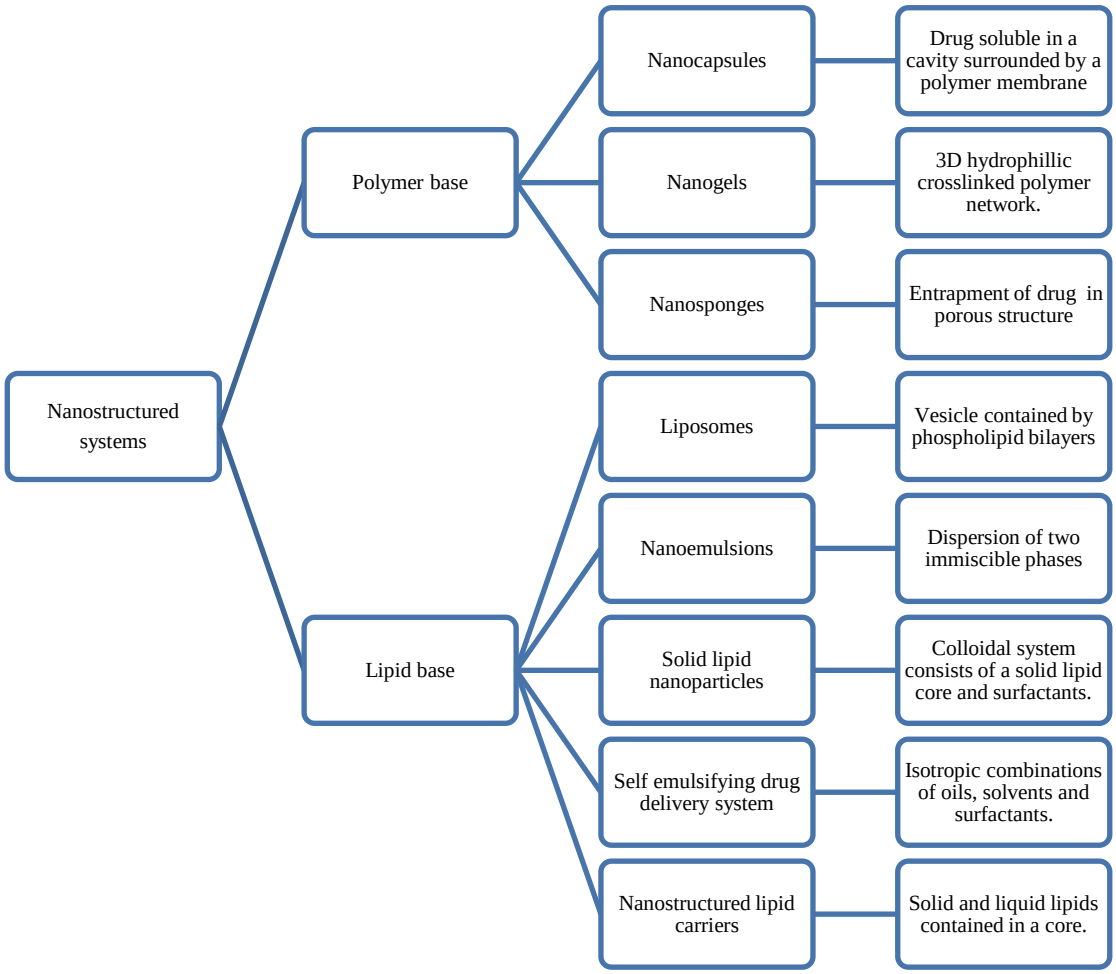


Figure 4.1: Types of nanostructured systems available for essential oil formulation development.

4.2.1. Nanoemulsions

Emulsions are divided into microemulsions, coarse emulsions and nanoemulsions based on differences in droplet sizes and stability (Aswathanarayan and Vittal, 2019). Nanoemulsions are defined as a mixture of two immiscible liquids in which stability is achieved with a

surfactant. The liquid forms the continuous phase while sphere droplets form the dispersed phase. An oil dispersed in an aqueous emulsion is referred to an oil-in-water (o/w) emulsion, which is commonly used for the delivery of hydrophobic drugs. Nanoemulsions are suitable for hydrophobic and poorly soluble essential oils due its enhanced solubilization properties, which usually consist of small droplets in the size range of 20-200 nm (Taleb et al., 2018). Figure 4.2 illustrates the structure of nanoemulsions.

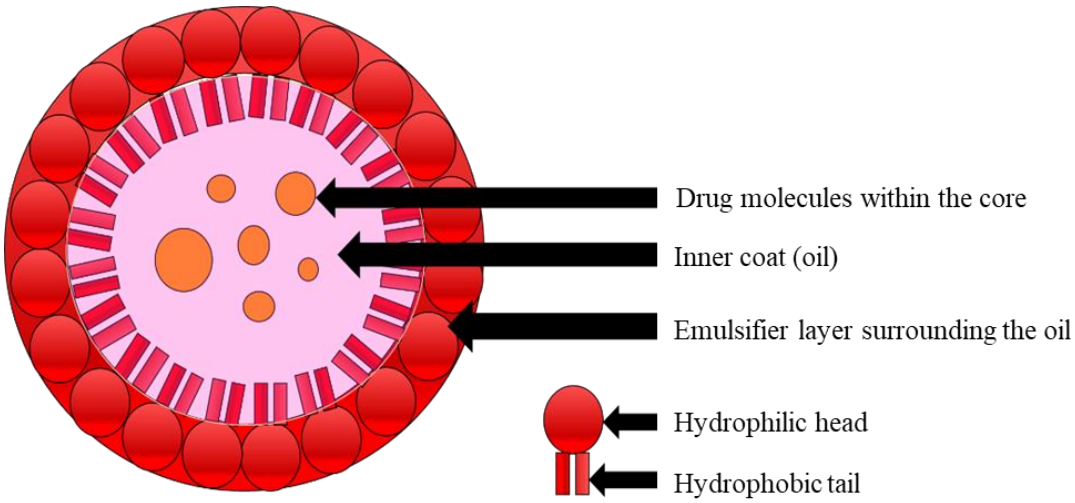


Figure 4.2: Structure of nanoemulsions

Nanoemulsions have advantageous properties, which include small droplet sizes that enables uniformity and decreases the risk of creaming and flocculation thus prolonging product shelf-life (Scholz and Keck, 2015, Sugumar et al., 2015). Nanoemulsions have kinetic stability and are not generally impacted by temperature or pH variations. Therefore, decreased amounts of surfactants are required to maintain stability compared to other types of emulsion formulations (Aswathanarayan and Vittal, 2019). The narrow range of droplet sizes can also prevent Ostwald ripening which is a physical instability where larger oil globules increase in size at the cost of smaller globules (Scholz and Keck, 2015). In terms of antimicrobial activity, a smaller droplet diameter can be linked to a broader spectrum of activity against pathogens, which is advantageous for application against respiratory tract infections (Sugumar et al., 2015). The limitation of nanoemulsions is the inherent low viscosity of the formulation which can be mitigated by employing certain thickening agents such as polymers in hydrogel thickened nanoemulsions (Barradas et al., 2018). A summary of the formulation characteristics required for an optimum nanoemulsion formulation can be seen in Table 4.2.

Table 4.2: Summary of the optimum ranges for nanoemulsion formulation

Nanoemulsion characteristic	Optimal range
Particle size	< 100 nm
Polydispersity index (PDI)	0.20 – 0.50
Particle size post centrifugation	< 100 nm
Polydispersity index (PDI) post centrifugation	0.20 – 0.50

4.2.2. Optimized essential oils for formulation design

Based on the predicted synergy results of SynergyFinder and the isobolograms, an optimum combination of essential oils for antimicrobial synergy was identified. The antimicrobial interaction of *Hyssopus officinalis* (hyssop) essential oil in combination with *Rosmarinus officinalis* (rosemary) essential oils against the nine respiratory pathogens was investigated using SynergyFinder 2.0. According to the synergy maps derived, an average synergy score of 9.76 was determined against all nine micro-organisms. A borderline synergistic result indicative of broad-spectrum antimicrobial effect, and the highest overall score of all five combinations of essential oils studied using variable ratio analysis. The results of SynergyFinder established that the optimal blend of each essential oil comprises of 49.57% of *H. officinalis* to 50.43% of *R. officinalis* to achieve an average synergy score ranging from 14.25 to 57.14 against the pathogens studied. This optimized blend was then formulated into a nanoemulsion, using the self-emulsification technique. The findings of this are reported in **Article 5**.

Article 5 - Original article on essential oil formulation design

At the time of this thesis, this article had been submitted for publication (05 October 2022) in the Journal of Essential Oil Research. This article is shown as it appears in submission.

Article

Designing an ideal essential oil combination for nanoemulsion formulation

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Designing an ideal essential oil combination for nanoemulsion formulation

ABSTRACT

Essential oils are well studied for their antimicrobial effects; however, research on blends extending to formulations are rarely scientifically explored and validated. This study aimed to quantify and optimize the synergy of an essential oil blend by means of computational interpretation to create a nanoemulsion formulation ideal for respiratory inhalation. The nanoemulsion blend consisted of essential oils from *Hyssopus officinalis* var. *angustifolius* (M.Bieb.) Benth. (hyssop) in combination with *Rosmarinus officinalis* var. *angustifolius* (Mill.) DC. (rosemary). The prediction tool, SynergyFinder was implemented to determine optimal synergy blends for these essential oils. According to the synergy maps derived, an optimal blend of each essential oil comprises of 49.57% of *H. officinalis* to 50.43% of *R. officinalis*. This optimized blend was then formulated into a nanoemulsion, using the two-component, self-emulsification technique. The essential oil nanoemulsion showed strong *in vitro* antimicrobial activities against pathogens of the respiratory tract, including; *Streptococcus pneumoniae* (ATCC 49619), *Haemophilus influenzae* (ATCC 19418), *Klebsiella pneumoniae* (ATCC 13883) and *Moraxella catarrhalis* (ATCC 23246) with an average 6-fold improvement in antimicrobial effect when compared to the neat essential oils assayed individually. The blended *H. officinalis* and *R. officinalis* essential oil nanoemulsion was validated in this study and therefore holds potential to be developed as a natural antimicrobial agent for the management of respiratory tract infections.

KEYWORDS: respiratory infections, antimicrobial, SynergyFinder, nanoemulsion, combination, essential oil.

1. Introduction

Essential oils have been used for their antimicrobial properties as early as 4500 BC, where the Ancient Egyptians made use of these oils in cosmetics and ointments for medicine (1). As a result of the empirical knowledge preserved for thousands of years on the antimicrobial potential of essential oils, research performed in laboratories globally have substantiated these effects, thus promoting the application of essential oil-containing pharmaceutical products (2). Despite the antimicrobial and other pharmacological properties associated with essential oils, their pharmaceutical use is often limited due to challenges in formulation development. Some limitations for the development of essential oils into pharmaceutical products include physical-chemical properties (e.g. poor water solubility), toxicity and environmental instabilities due to heat, oxygen and moisture (3). Essential oils are hydrophobic (which is a limitation for inclusion in some delivery systems), however, novel pharmaceutical technology can be employed to improve the inclusion capabilities of essential oils in formulations (4, 5). According to the literature, oil-in-water (O/W) nanoemulsions are especially suitable for the delivery of essential oils, due to their oil-based core, high-loading capability, and the large variety of available emulsifiers and stabilizers available for use (6, 7). Previous studies incorporating essential oils into nanoemulsion systems for antimicrobial purposes have provided promising results (8, 9). An earlier study Sugumar, Ghosh (10), aimed to determine the antimicrobial effect of the essential oil of *Eucalyptus globulus* Labill. (Eucalyptus) when encapsulated into a nanoemulsion system. The nanoemulsion demonstrated a greater antimicrobial effect against *Staphylococcus aureus* than when compared to the essential oil alone. Further studies have demonstrated similar improved antimicrobial effects (2, 11). Much of the work produced concerning essential oils incorporated into nanoemulsion formulations have considered the effect of the essential oil when used alone (12-18). Despite the positive findings that support the potential use of these individual essential oils in nanoformulations for

antimicrobial purposes, the practice of aromatic medicine predominantly makes use of essential oils in combination with other essential oils to achieve an enhanced therapeutic effect (19, 20). A limited number of studies exist that have explored the effects of essential oils in combination when formulated as nanoemulsions for antimicrobial purposes (21-25). Much of the published findings are limited to essential oils used against pathogens associated with food spoilage. The rationale for combination use for synergistic antimicrobial purposes within a pharmaceutical formulation is to encourage the use of lower doses of each component while maintaining optimized antimicrobial effects (21, 22). In order to optimize antimicrobial activity of an essential oil combination for formulation development, it is advisable to use an experimental design to define the most suitable proportions of each ingredient (oil) in an effective mixture (22). The use of computer-aided software to quantify synergy for drug design and development has been in use within the pharmaceutical industry for a number of years, with great success (26). In pre-clinical formulation studies concerning a combination of drugs, functional screening assays that probe the effects of the drug combination as per a dose-response matrix assay are commonly employed (27). The use of this technology aims to develop innovative formulations based on optimized interactions and is considered the favored method for interactive assessment in formulation design (21).

In our previous studies (28, 29) we provided results of the antimicrobial potential of essential oils alone and in equal combinations against nine pathogens of the respiratory tract. Based on the findings of these studies, one unique combination comprising of the essential oils *Hyssopus officinalis* var. *angustifolius* (M.Bieb.) Benth. (hyssop) and *Rosmarinus officinalis* var. *angustifolius* (Mill.) DC. (rosemary) was identified as the most promising for use, having broad-spectrum antimicrobial activity, anti-inflammatory and non-toxic effects when used in equal ratio combinations (28, 29). This study explored this unique combination of essential oils

to quantify the optimum essential oil dose ratio that would provide antimicrobial synergy and further explored the combination as an antimicrobial nanoformulation.

2. Materials and methods

2.1. Materials

One essential oil combination was selected for this study and included *H. officinalis* var. *angustifolius* in combination with *R. officinalis* var. *angustifolius* (These essential oils were procured from a flavor and fragrance industry provider, Prana Monde (Hainaut, Belgium). The two essential oils used in this study have been previously chemically characterized using gas chromatography coupled with a mass spectrometer (GC-MS) and reported in our earlier studies (28, 29). The major chemical constituents of these oils are provided in the Supplementary data (Table S1). The primary role of the surfactant within the formulation is to stabilize the emulsion by reducing surface tension at the oil/water interface (30, 31). Due to the lipid nature of essential oils, Caprylocaproyl macrogol-8-glyceride (Labrasol) was selected as the surfactant for the formulation. Labrasol is a non-ionic water-dispersible surfactant specifically used in lipid-based formulations to solubilize poorly water-soluble ingredients. Labrasol was obtained as a donation from Gattefossé (Saint-Priest, France). Propylene glycol (Sigma-Aldrich) was selected as a co-surfactant to the formulation as a means to provide further stability to the essential oil nanoemulsion.

2.2. Preparation of cultures

Microbial cultures were selected based on their relevance to respiratory infections and included the Gram-positive strains *Staphylococcus aureus* (ATCC 25924), *Streptococcus agalactiae* (ATCC 55618), *Streptococcus pneumoniae* (ATCC 49619) and *Streptococcus pyogenes* (ATCC 12344). The Gram-negative strains included *Haemophilus influenzae* (ATCC 19418),

Klebsiella pneumoniae (ATCC 13883) and *Moraxella catarrhalis* (ATCC 23246). The non-pathogenic *Mycobacterium* strain *M. smegmatis* (ATCC 19420), and yeast strain *Cryptococcus neoformans* (ATCC 14116) were also included. All cultures were prepared as per our earlier studies (28, 29). A waiver for the use of these micro-organisms was granted by the University of the Witwatersrand Human Research Ethics Committee (Reference W-CJ-160720-2).

2.3. Antimicrobial combination optimization using SynergyFinder (version 2.0)

SynergyFinder (Version 2.0) facilitates the analysis of drug combination experiments and provides an interface for visualizing the drug combination landscapes in an interactive manner (27). The essential oil combination selected was investigated at varying ratios (Table 1) as a means to complete a dose-response matrix for evaluation by the SynergyFinder software, using the broth microdilution assay as previously reported (32).

Table 1. The concentrations of each essential oil within the essential oil blend

Combination	Concentrations of essential oils in combination (µL)	
	H. officinalis	*R. officinalis*
1	0	100
2	0	25
3	0	50
4	0	75
5	10	90
6	20	80
7	25	0
8	25	75
9	30	70
10	33	66
11	40	60
12	43	57
13	50	0
14	50	50
15	57	43
16	60	40
17	66	33
18	70	30

19	75	0
20	75	25
21	80	20
22	90	10
23	100	0

The 96-well micro-titre plates were prepared by aseptically adding 100 µL of sterile broth, respective for each micro-organism tested, into each of the wells. A stock concentration of each essential oil (32 mg/mL in acetone) was added to the first row at varying ratios (Table 1). The essential oil combinations were then serially diluted to concentrations ranging from 8.00 to 0.06 mg/mL descending down each column of the micro-titre plate. A 100 µL volume of positive, negative and culture control was included for each micro-organism. The positive controls (Sigma–Aldrich) comprising of 0.01 mg/mL ciprofloxacin (for bacteria) or 0.10 mg/mL amphotericin B (for the yeast) were included to ensure microbial susceptibility. The negative control of 32 mg/mL water in acetone was included to exclude the attribution of antimicrobial effects due to the solvent. The culture controls comprised of growth medium with the relevant pathogen to ensure that the appropriate broth supported microbial growth. An inoculum concentration of approximately 1×10^6 colony forming units per mL (CFU/mL) was prepared for each micro-organism studied and 100 µL of the inoculum added to each well. Before incubation, each micro-titer plate was sealed with sterile adhesive sealing film. Following incubation, 40 µL of 0.04% w/v *p*-iodonitrotetrazolium violet solution (INT; Sigma-Aldrich) was added into each well of the micro-titer plate in order to determine micro-organism viability. A change of color in the well from clear to a purple-pink indicates microbial growth, therefore the lowest concentration displaying no color change was recorded as the MIC. Each experiment was repeated in triplicate and the mean value was recorded.

The data generated from the varied ratio combination MIC assay was entered into SynergyFinder 2.0, a stand-alone web-application for interactive analysis and visualization of the essential oil combination screening data (33). A log-logistic model was applied to the varied

ratio MIC findings in order to generate dose-response curves for the essential oils in each combination. Essential oil combination antimicrobial responses were then plotted as heat maps to identify the concentrations at which the essential oils used in combination had maximum inhibitory effect on microbial growth. The degree of synergy per essential oil combination was analyzed using the response surface model based on the Bliss reference synergy model. The synergy score determined for the essential oil combination investigated was averaged over all the dose combination measurements, and a 3D synergy map was generated that highlights synergistic and antagonistic dose regions in red and green colours, respectively. On interpretation, the summary synergy scores of less than 10 was considered antagonistic, a range from −10 to +10 as additive, and greater than +10 as synergistic (34).

2.4. Preparation of nanoemulsion

The essential oil nanoemulsion was prepared using the two-component, self-emulsification method. Labrasol was first mixed with propylene glycol and stirred magnetically (IKA Plate, RCT digital, Deutschland, Germany) for 5 min at a speed of 500 rpm. The essential oils were subsequently added to produce a formulation of a final concentration of 2.5% ($^{v}/_{v}$) to the Labrasol and propylene glycol mixture and stirred for a further 15 min. Sterile distilled water (30 mL) was then added dropwise and stirred magnetically until a homogeneous translucent appearance was achieved. The completed formulation was then stored in sealed amber bottles to prevent any loss of essential oil due to evaporation.

2.5. Characterization of the nanoemulsion

The physical attributes of a nanoemulsion formulation determines the formulation stability and efficacy as well as its *in vitro* behaviour (35). These physical attributes of importance include the average particle size distribution and PDI. The particle size distribution, and PDI of the

samples were determined using dynamic light scattering (DLS) at 25°C using a zeta-potential and particle size analyzer (ELSZ-2000, Otsuka Electronics Co., Ltd. Japan). In the case of pulmonary drug delivery for systemic absorption, aerosols with a small particle size are required to ensure peripheral penetration (35, 36). Particles smaller than 3 μm have an approximately 80% probability of reaching the lower airways while around 50–60% of these particles will be deposited in the alveoli (37), and hence this was a parameter for formulation.

2.6. Stability of the nanoemulsion

2.6.1. Centrifugation stability

The essential oil nanoemulsion was centrifuged at 5000 rpm for 30 min to evaluate the stability. Stability was investigated by storing 5 mL of the nanoemulsion at 4 ± 1 °C, 25°C ± 1°C, 37°C ± 1°C and 60°C ± 1°C in duplicate for one month (30). The samples were observed for instability including, creaming or flocculation.

2.7. Antimicrobial activity of the nanoemulsion

The broth microdilution method (38), was used to quantify the antimicrobial inhibitory activity of the formulated essential oil nanoemulsion against pathogens selected on their relevance to respiratory infections. The micro-titre plates were prepared aseptically, as per the antimicrobial studies undertaken for the combination optimization using SynergyFinder (Version 2.0). Variations to the method included the addition of 100 μL of the essential oil nanoemulsion, added to the first row. The positive controls remained 0.01 mg/mL ciprofloxacin (for bacteria) or 0.10 mg/mL amphotericin B (for the yeast) to ensure microbial susceptibility. The negative control of 15% ($^{v}/_{v}$) Labrasol and 7% ($^{v}/_{v}$) propylene glycol in 30 mL of distilled water was included to exclude the attribution of antimicrobial effects due to the formulation solution base. The culture controls comprised of growth medium relevant to the pathogen studied with the

relevant pathogen to ensure that the appropriate broth supported microbial growth. Following incubation, 40 μL of 0.04% $^{w}/_{v}$ *p*-iodonitrotetrazolium violet solution (INT; Sigma-Aldrich) was added into each well of the micro-titer plate in order to determine the MIC. Each experiment was repeated in triplicate and the mean value was recorded.

3. Results and discussion

3.1. Antimicrobial optimization

The antimicrobial interaction of *H. officinalis* essential oil in combination with *R. officinalis* essential oils against a selection of nine respiratory pathogens was investigated using SynergyFinder 2.0. (Figure 1).

According to the synergy maps, an average synergy score of 9.76 was determined against all nine pathogens studied. The highest synergy was noted against *H. influenzae* for which a synergy score of 27.621 was noted, with greater synergy assigned to ratios higher in *H. officinalis* than *R. officinalis*, while optimal synergy was determined for each essential oil in equal ratios of this combination. Synergy was also noted against *S. pyogenes*, *M. smegmatis* and *C. neoformans* with synergy scores of 10.470, 11.864 and 13.263, respectively. Marginal synergy was determined for the combination against the pathogen *S. aureus* (synergy score = 9.505), while additive scores were noted against all other pathogens with scores of -0.029 (*S. agalactiae*), 7.602 (*S. pneumoniae*), 3.763 (*K. pneumoniae*) and 3.780 (*M. catarrhalis*) measured. The results of SynergyFinder established that the optimal blend of each essential oil comprises of 49.57% of *H. officinalis* to 50.43% of *R. officinalis* to achieve an average synergy score ranging from 14.25 to 57.14 against the pathogens studied.

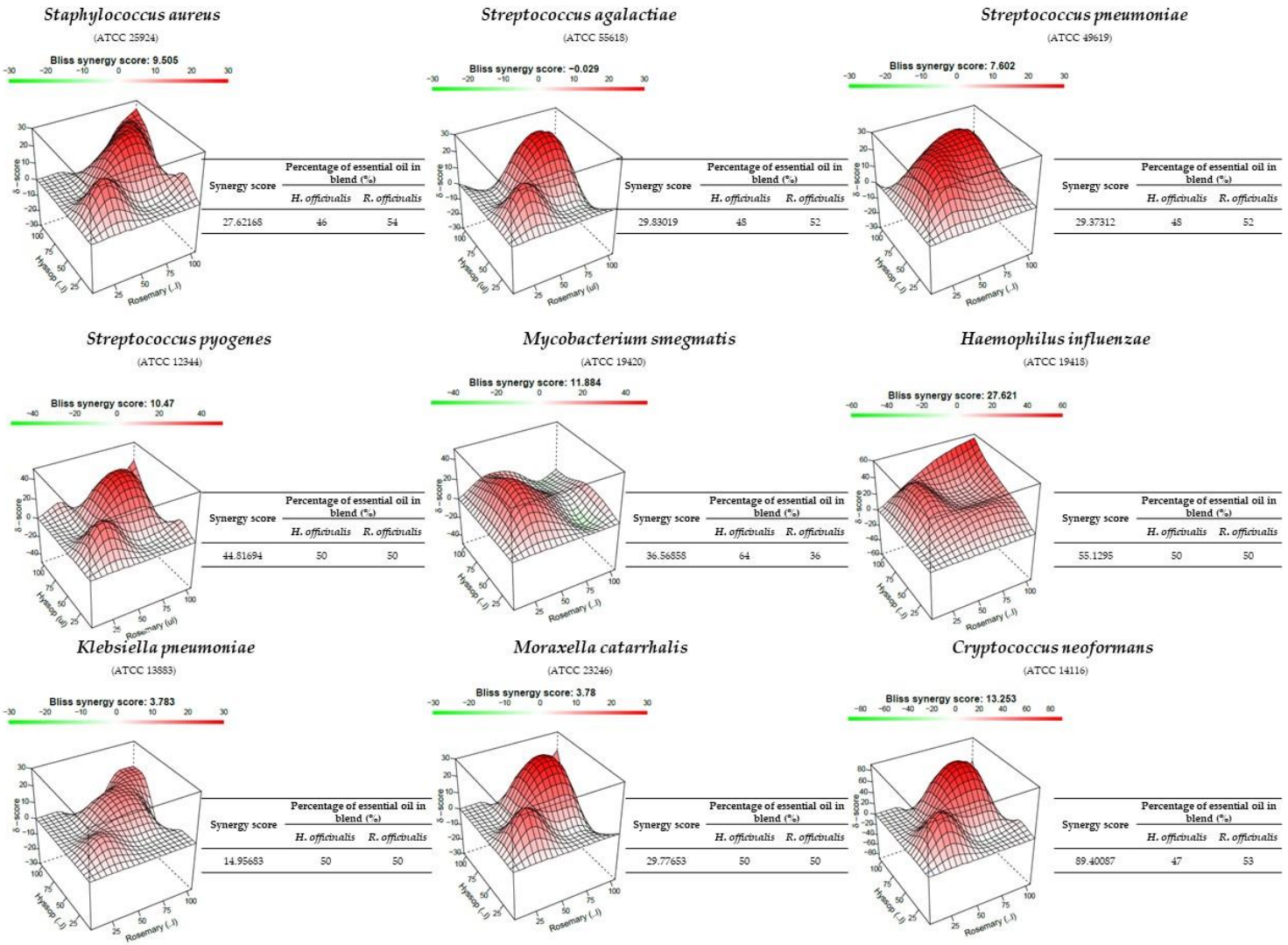


Figure 1. Visual representations of the antimicrobial interaction of the combination of *H. officinalis* and *R. officinalis* essential oils against nine micro-organisms responsible for respiratory tract infections. Positive (red regions) of the response surface plot indicate synergistic effects; while negative (green regions) indicate antagonistic effects. Optimal ratios of *H. officinalis* and *R. officinalis* essential oils are indicated in adjacent tables, per micro-organism.

3.2. Characterization of the nanoemulsion

The blended *H. officinalis* and *R. officinalis* essential oil nanoemulsion was stable to coalescence and Ostwald ripening. The average particle size of the blended *H. officinalis* and *R. officinalis* essential oil nanoemulsion was determined as 47.89 nM using a zeta-potential and particle size analyzer (Figure 2).

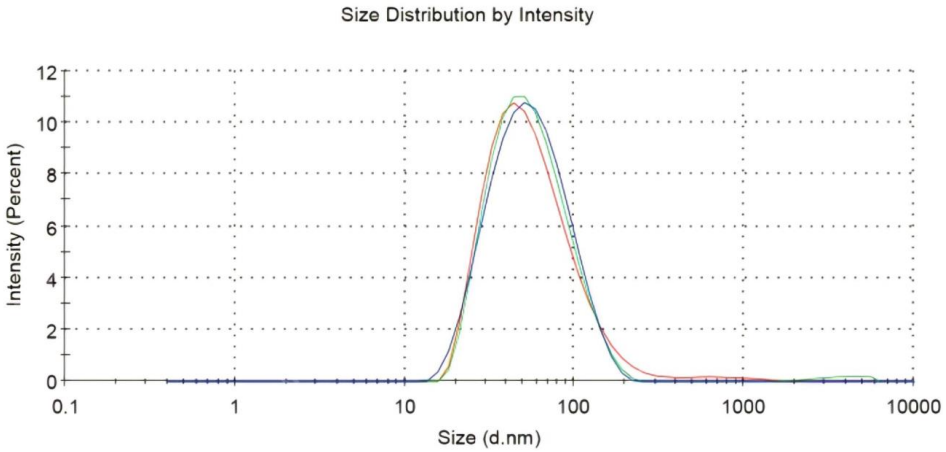


Figure 2. Particle size of the essential oil nanoemulsion as determined by zeta-potential and particle size analyzer.

Oil in water emulsions are classified based on the size of the droplet, with nanoemulsions classified for droplet sizes of less than 100 nM (39). Therefore, these findings indicate the emulsion was within nano-range. Other studies incorporating the use of essential oils in nanoemulsions have defined a range of nanodroplet sizes between 23.4 and 100 nM with droplet sizes varying dependent on the constituents selected, operating conditions, and preparation methods used (40-43). The polydispersity index (PDI) value provided by the nanosizer informs if the nanoemulsion droplets are well dispersed with a measure of 0.20 to 0.50 expected for pharmaceutical preparations (44). The PDI value for this study was determined as 0.202, which is desirably low and suggestive of well-dispersed nanoemulsion.

3.3. Stability of the nanoemulsions

3.3.1. Centrifugation stability

The appearance of the blended *H. officinalis* and *R. officinalis* essential oil nanoemulsion following formulation provided a clear and transparent liquid, without observed turbidity or precipitate (Figure 3A). After centrifugation at 5000 rpm for 30 min, the formulation maintained the same clear and transparent appearance (Figure 3B). The particle size of the nanoemulsion droplets were again measured using a zeta-potential and particle size analyzer. The droplet sizes were maintained after the centrifugation with droplet sizes slightly reduced to 37.55 nM and a PDI of 0.189, indicating good stability.

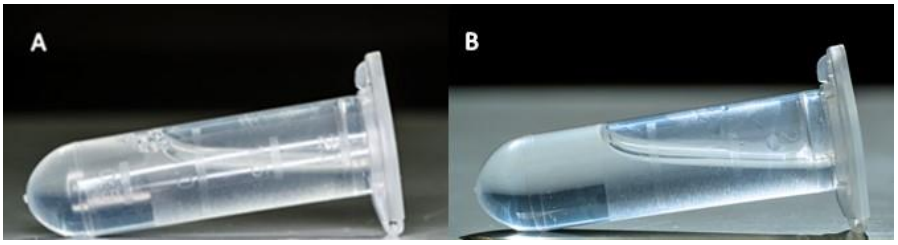


Figure 3. The appearance of the blended *H. officinalis* and *R. officinalis* essential oil nanoemulsion following formulation (A) and following centrifugation (B).

The nanoemulsion, stored at 4 ± 1 °C, 25 ± 1 °C, 37 ± 1 °C and 60 ± 1 °C for one month, respectively showed no phase separation or creaming properties at all temperatures, indicating stability of formulation (Figure 4).



Figure 4. The appearance of the blended *H. officinalis* and *R. officinalis* essential oil nanoemulsion (in duplicate) following storage at 4 ± 1 °C, 25 ± 1 °C, 37 ± 1 °C and 60 ± 1 °C for one month.

3.4. Antimicrobial validation of the formulation

The blended *H. officinalis* and *R. officinalis* essential oil nanoemulsion was investigated against nine pathogens of the respiratory tract by means of minimum inhibitory concentration (MIC) analysis. A noteworthy antimicrobial effect was determined for the blended *H. officinalis* and *R. officinalis* essential oil nanoemulsion against all nine respiratory pathogens with MIC values of 0.07 to 1.17 mg/mL (mean of 0.36 mg/mL) observed (Table 2). The measured MICs of the neat, combined essential oils prior to formulation against the nine pathogens studied was on average 2.17 mg/mL (29). The nanoemulsion exhibited better inhibitory effects across all pathogens studied, with a six fold increase in antimicrobial activity than when the neat essential oils when blended.

Table 2. The mean MIC in mg/mL (n = 3) value of the blended *H. officinalis* and *R. officinalis* essential oil nanoemulsion investigated against test pathogens

	Neat essential oils in combination (29)	Essential oil nanoemulsion	Ciprofloxacin (positive control)	Amphotericin B (positive control)	Formulation Solution Base (negative control)
Staphyloccocus aureus (ATCC 25924)	2.00	0.07	0.50×10^{-3}	NA	> 4.93
Streptococcus agalactiae (ATCC 55618)	2.00	0.29	0.50×10^{-3}	NA	> 4.93
Streptococcus pneumoniae (ATCC 49619)	3.00	1.17	0.50×10^{-3}	NA	> 4.93
Streptococcus pyogenes (ATCC 12344)	1.00	0.29	0.50×10^{-3}	NA	> 4.93
Mycobacterium smegmatis (ATCC 19420)	1.50	0.29	0.50×10^{-3}	NA	> 4.93
Haemophilus influenzae (ATCC 19418)	4.00	0.29	0.25×10^{-3}	NA	> 4.93
Klebsiella pneumoniae (ATCC 13883)	4.00	0.59	1.00×10^{-3}	NA	> 4.93
Moraxella catarrhalis (ATCC 23246)	2.00	0.15	0.50×10^{-3}	NA	> 4.93
Cryptococcus neoformans (ATCC 14116)	0.09	0.07	NA	0.50×10^{-3}	> 4.93

The findings of this study are congruent with previous studies that showed that the conversion of essential oils into nanoemulsions having an improved antimicrobial effect. A previous study

(45), aimed to create an essential oil nanoemulsion containing *Cymbopogon flexuosus* (Nees ex Steud.) Will. Watson for antimicrobial use. The neat oil of *C. flexuosus* showed activity against *Candida albicans* at a concentration of 1.22 mg/mL while the nanoemulsion of the essential oil demonstrated an MIC of 0.28 mg/mL. This improved antimicrobial effect was also noted against the micro-organisms *Cryptococcus grubii* (MIC of neat oil was 0.58 mg/mL and the nanoemulsion was 0.28 mg/mL) and *Pseudomonas aeruginosa* (no antimicrobial effect by neat oil, and the nanoemulsion showed potential bactericidal activity at concentrations above 11.33 mg/mL). These findings are further supported by previous essential oil studies, e.g. *Origanum vulgare* L. (46) and *Thymus daenensis* Čelak (47). It has been noted that this enhanced antimicrobial effect noted by essential oils when encapsulated into a nanoemulsion is due to the nanometric size and resultant improved diffusion of nanoemulsions (48). These enhanced characteristics offered by nanoformulations provides opportunity for further exploration of varying essential oil blends.

4. Conclusions

This study is the first to apply SynergyFinder software for the visual interpretation of antimicrobial synergy between the essential oil combination *H. officinalis* in combination with *R. officinalis*. The essential oil combination of *H. officinalis* and *R. officinalis* demonstrated broad-spectrum antimicrobial potential with a Bliss model synergy score of 9.76. As a result of this potential, a nanoemulsion formulation was created for a blend of *H. officinalis* and *R. officinalis* essential oils at optimized ratios as per the identified synergy maps for nine respiratory associated pathogens. At the optimal concentration of the blended *H. officinalis* and *R. officinalis* essential oil nanoemulsion, the average particle size of the nanoemulsion was 47.89 nM and PDI 0.202. The nanoemulsion maintained good stability and dispersion after centrifuging at 5000 rpm for 30 min, stored at 60 ± 1 °C for one month. Furthermore, at

optimum concentrations, the blended *H. officinalis* and *R. officinalis* essential oil nanoemulsion showed noteworthy antimicrobial activity against all nine pathogens studied. Aerosolised nanoemulsions are promising alternatives for non-invasive drug delivery to the respiratory tract due to their improved and targeted antimicrobial approach. These characteristics of a nanoemulsion system and the proven enhanced interactions of the blended *H. officinalis* and *R. officinalis* essential oil nanoemulsion against pathogens of the respiratory tract further provide a rational for the potential of this blend for therapeutic use. This research indicates the potential for optimized essential oil combinations to be identified using a computational software workflow; and further formulated into nanosystems for targeted antimicrobial effects. As of now, research on the application of essential oils into nanoemulsions as antimicrobial agents are in fast-growing phase, however, there is a paucity in literature on the use of essential oils in combination. Advancements in this field of research require further exploration for commercial consideration and potential product development.

Supplementary Materials: Table S1: Identified major essential oil chemical constituents.

Acknowledgments

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Word count: 5499

CHAPTER 5:

SUMMARY AND CONCLUSION



5.1. Summary

A review of the aroma-therapeutic literature identified 55 essential oils for use in the respiratory tract (Leigh-de Rapper and van Vuuren, 2020). This study procured and investigated the chemical profiles and antimicrobial activity of 49 essential oils against pathogen reference strains, directly related to infections of the respiratory tract. Chemometrics, a multivariate tool, allowed for the review of the essential oil chemistry in tandem with the identified antimicrobial activity as a means to identify structure-activity relationships. This tool allowed for the identification of the compounds responsible for antimicrobial activity, proving it to be a useful tool in identifying patterns of compounds responsible for the antimicrobial effects of essential oils (Rapper et al., 2021). Eugenol was identified as the most frequently reoccurring biomarker that contributed to broad-spectrum noteworthy antimicrobial activity of essential oils; while *p*-Cymene and cis-β-pinene were frequently responsible for poor antimicrobial activity.

Essential oil combination studies were undertaken to review the antimicrobial and potentially toxic effects of essential oils when placed in 1:1 blends. A summary of these interactions for these combinations, as classified according to the criteria of synergistic, antagonistic, additive or non-interactive, and can be viewed in Figure 5.1.

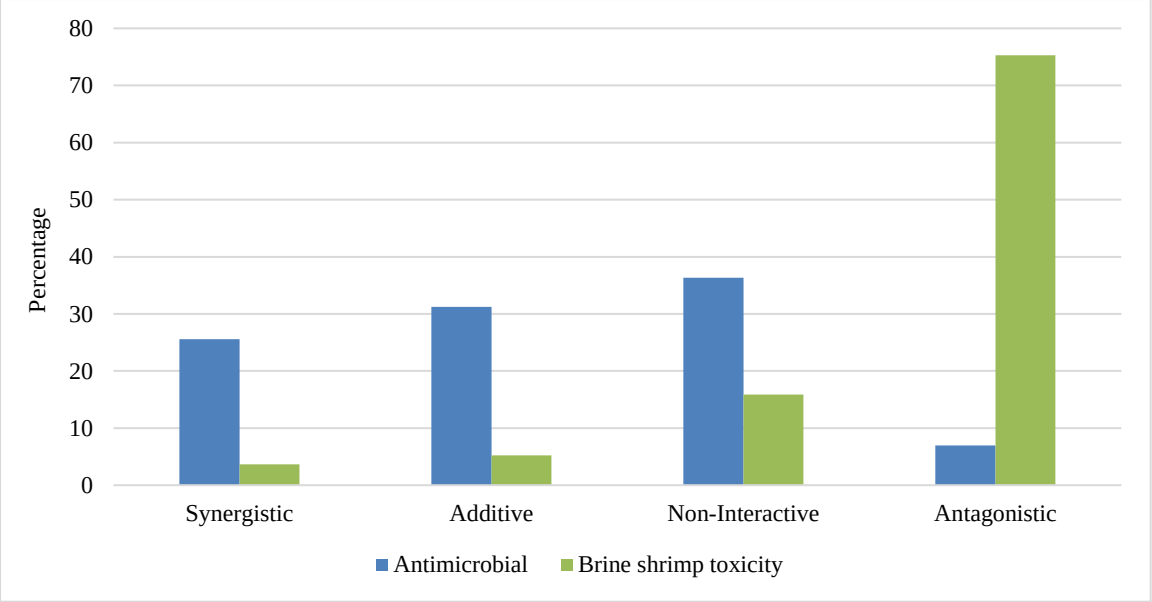


Figure 5.1: Percentage interactions of 1:1 ratio combinations of essential oils for antimicrobial and non-toxic effects against brine shrimp.

Interestingly, the number of synergistic interactions for antimicrobial effects appears substantial; however, toxic effects predominate in antagonistic potential (more toxic effects) amongst these blends. However, not all blends that were antagonistic (more toxic) in effect against brine shrimp were considered lethally toxic. Approximately 31.0% of the combinations studied demonstrated non-toxic effects against the brine shrimp, compared to the 20.4% when the essential oils were studied independently. From these results, it appears that the essential oils show the potential to quench toxicity when used in combination.

Further investigation of the antimicrobially synergistic and non-toxic to brine shrimp essential oil blends was undertaken to determine the potential of these combinations for anti-inflammatory and non-toxic effects against lung cell lines. A summary of these interactions for these combinations is given in Figure 5.2.

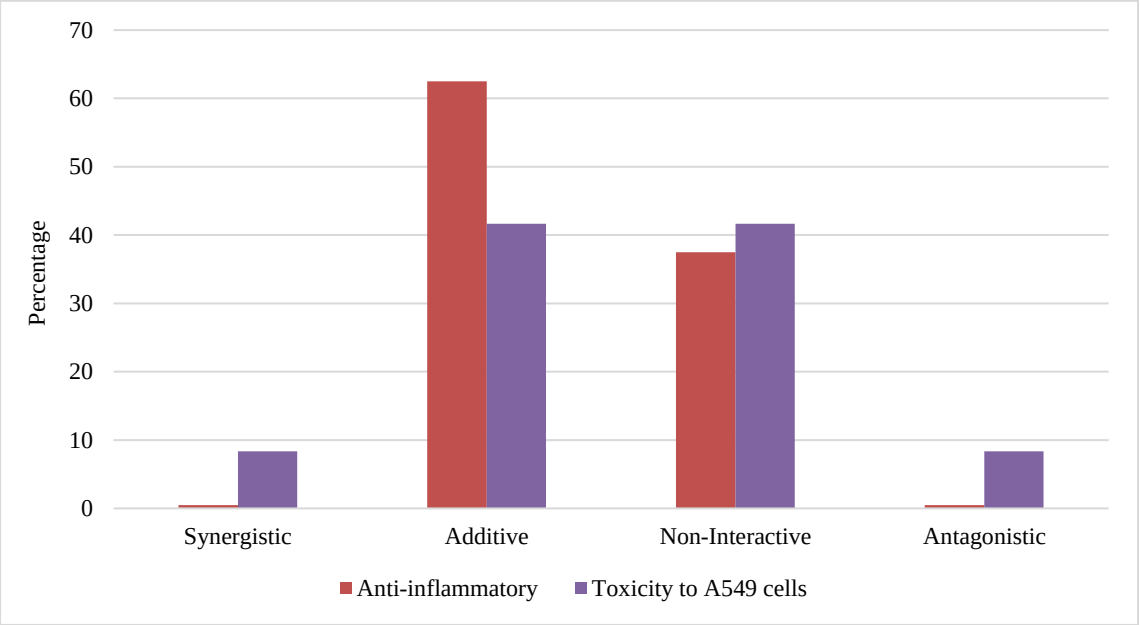


Figure 5.2: Percentage interactions of 1:1 ratio combinations of antimicrobially synergistic essential oils for anti-inflammatory and non-toxic effects against A549 lung cells.

The antimicrobially synergistic and non-toxic to brine shrimp essential oil blends demonstrated positive anti-inflammatory outcomes with 62.5% of interactions proving an additive effect. Five distinct combinations (*Cupressus sempervirens* in combination with *Melaleuca alternifolia*, *Hyssopus officinalis* in combination with *Rosmarinus officinalis*, *Origanum marjorana* in combination with *M. alternifolia*, *Myrtus communis* in combination with *M. alternifolia* and *Origanum vulgare* in combination with *M. alternifolia*) were found to be the

most promising, demonstrating antimicrobial activity, reduced cytotoxicity and improved anti-inflammatory effects. The potential for varied antimicrobial activity of these five essential oil combinations was investigated in varied ratios. The interactions between essential oil combinations was analysed using isobolograms and SynergyFinder 2.0 software to visualize the synergistic potential. A summary of these interactions for these varied combinations is given in Figure 5.3.

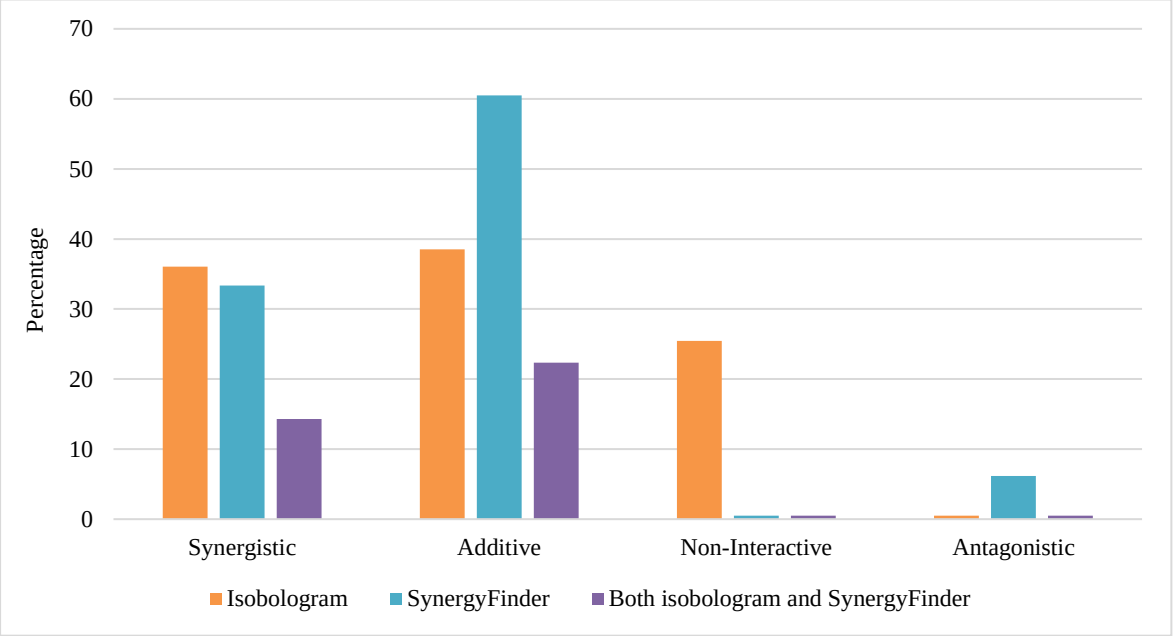


Figure 5.3: Percentage interactions of the varied ratios for combinations as per isobologram and SynergyFinder analysis

Inconsistencies in interpretation of antimicrobial effect was noted between interaction analysis (isobolograms and SynergyFinder) which is not an uncommon finding (Baeder et al., 2016, Meyer et al., 2020). If both the SynergyFinder and the isobologram classify a ratio as synergistic, then the interaction is deemed strongly synergistic; whereas if the ratio is classified as synergistic according to one model only, then the interaction is weakly synergistic (Tang et al., 2015). With this in mind, 15.7% of synergistic interactions were deemed strongly synergistic, while a further 25.3% of ratios were noted as weakly synergistic. The essential oil combination of *H. officinalis* and *R. officinalis* demonstrated broad-spectrum antimicrobial potential with a Bliss model synergy score of 9.76 at ratios of between 49.57% of *H. officinalis* to 50.43% of *R. officinalis*. The findings of this study proved that isobolograms and computational modelling tools such as SynergyFinder can work collaboratively to pin-point optimum antimicrobial potential.

The use of computer-aided software to quantify synergy and optimize ingredients within a pharmaceutical product is an important step in the drug design process, as it aims to reduce cost while maintaining pharmacological effects. Therefore, if an essential oil formulation were to be created for commercial consideration, advancements in this field of research would be required to confirm essential oil synergy in combination. With this in mind, an essential oil nanoemulsion comprising *H. officinalis* and *R. officinalis* was created. The formulation met qualifying criteria for a suitable nanoemulsion. Furthermore, the blended *H. officinalis* and *R. officinalis* essential oil nanoemulsion showed noteworthy antimicrobial activity against all nine pathogens studied. A summary of these antimicrobial effects compared to the essential oil combination not in formulation can be viewed in Figure 5.4.

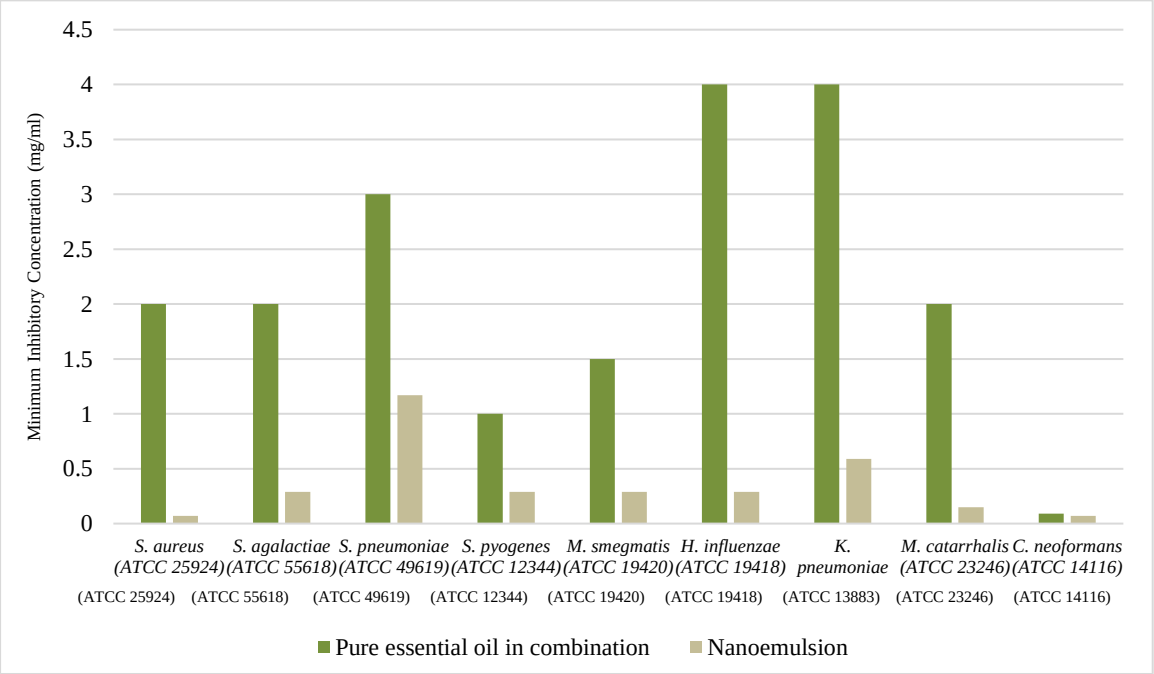


Figure 5.4: MIC values of the pure essential oils in combination compared to optimized essential oil blend in nanoemulsion formulation against pathogens of the respiratory tract.

The findings of this study demonstrated the potential to apply an optimized blend of an essential oil combination into an ideal and stable pharmaceutical formulation. This formulation also went on to prove the vast enhancement of essential oils when blended into formulation for antimicrobial effect.

5.2. Future Recommendations

5.2.1. *Antimicrobial studies*

Only reference strains pertaining to respiratory pathogens were used and therefore, future studies could consider the use of clinical strains, and resistant strains, for comparison. As previously mentioned, the same essential oil differs in chemistry dependent on a number of environmental factors. The antimicrobial differences between the varying essential oil chemotypes could be considered, alone and in combination. Antimicrobial research can also be extended to consider other clinical conditions of relevance. In addition, other methods of antimicrobial analysis could be considered such as vapour studies for the determination of essential oil MIC. Additional essential oils, not identified in this study, could be considered for future study.

5.2.2. *Pharmacological studies*

Anti-inflammatory and non-toxic effects against lung cells were only conducted for those combinations deemed to be synergistic in antimicrobial effect. The study could be expanded to consider these pharmacological effects on a larger number of essential oil combinations. In addition, the use of the isobolograms and SynergyFinder tools were also limited to antimicrobial effect and could be expanded to include anti-inflammatory and effects for the creation of a polygonogram for optimized clinical formulation.

5.2.3. *Combination studies*

According to the aromatherapeutic literature used to derive the combinations of essential oils for respiratory use, multiple ‘recipes’ exist that combine essential oils in blends of three to five essential oils. These combinations could be studied further for antimicrobial, anti-inflammatory and non-toxic effects; and compared to the efficacy of the single essential oils. In addition, the use of isobolograms and SynergyFinder could be applied to review the essential oils within these multi-component blends providing an enhanced effect. In addition, essential oils should be studied in combination with conventional antimicrobial agents as a means to enhance antimicrobial effects and reduce antimicrobial resistance. The use of essential oils in combination with antibiotics have shown to act as resistance modifying agents by reducing the dosage of antibiotics and improving effectiveness due to synergy with essential oils. This

strategy shows potential to reverse antimicrobial resistance and improve the effectiveness of currently available antimicrobial agents, and therefore is worthy of further study.

5.2.4. *Prediction tool analysis*

Varying prediction tools exist to establish synergy among drug combinations and include programmes such as DrugComb (Zheng et al., 2021) CompuSyn (Chou and Martin, 2006), ComBenefit (Di Veroli et al., 2016), DIGREM (Zhang et al., 2019), DeepSynergy (Preuer et al., 2018), SynPathy (Tang and Gottlieb, 2022) and SynergyFinder Plus (Zheng et al., 2022). Each method applies the same mathematical models for prediction; however, researchers have discovered variations in synergy prediction across programmes (Meyer et al., 2020). An expansion of this study would be to use multiple prediction tools to confirm optimal synergy amongst essential oil blends.

5.2.5. *Mechanism of action and metabolomics studies*

Numerous mechanisms of action have been proposed for different essential oils by researchers; however, much of the understanding is derived from studies that have tested purified molecules, and not whole oils (Buriani et al., 2020). This gap in research limits researcher's abilities to correlate these findings to a pharmacological effect of an essential oil. Despite this, more studies are required to elucidate essential oil mechanism of action in order to inform accurate optimization of essential oil blends for therapeutic use. Metabolomic studies can bridge this gap by providing the chemical fingerprint of an essential oil and characterizing this to physiological effect (Kharbach et al., 2020). In addition, metabolomics studies provide the complete set of metabolites produced within an organism, including description of enzymatic pathways and networks, which help to describe changes in microbial environments after exposure to an essential oil (Sieniawska and Georgiev, 2021). This is useful in in revealing the microbial mechanisms of resistance following exposure to essential oils (Tang, 2011, Sieniawska and Georgiev, 2021). Pathogenic mechanisms exist that prove resistance to essential oils including, selective membrane permeability, regulated efflux/influx and chemotaxis-controlled motility. These mechanisms can be overcome by use of a synergistic combination of essential oils that target varying mechanisms of action. These studies will help to inform which essential oils could be combined based on mechanism of action and potential to reduce microbial resistance.

5.2.6. *Essential oil compound studies*

Due to the complexity of essential oil chemistry and variability amongst chemotypes, studies considering the effects of essential oil compounds should be expanded on for application into pharmaceutical formulation. Chemometric analysis provided valuable information concerning putative biomarkers responsible for antimicrobial activity (Rapper et al., 2021). These compounds could be expanded to investigate anti-inflammatory and toxic effects, alone and in combination. Furthermore, these compounds could be further studied for optimization.

5.2.7. *In vivo and clinical studies*

The developed essential oil nanoemulsion was not assessed for clinical or *in vivo* validation and could therefore be considered for future study.

5.3. Final conclusion

This study aimed to investigate the accuracy of synergy prediction made by computer-aided software for the use of essential oils in the treatment of respiratory infections. In completing the objectives of this study, it was determined that prediction software, although critical in preclinical formulation design, faces many challenges and flaws in regards to practical translation of antimicrobial effects. These challenges have been overcome in this study and further prove the benefit of these tools to optimize essential oil combinations for antimicrobial effect. This study challenged conventional methods of antimicrobial analysis and was majorly successful. Yet, this research is only the beginning in identifying optimized blends of essential oils in the management of communicable infections by means of synergy prediction methods and thus sets the starting point for innovative, translational essential oil research of clinical relevance.

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APPENDICES

Appendix A – Details of authors who contributed to the project

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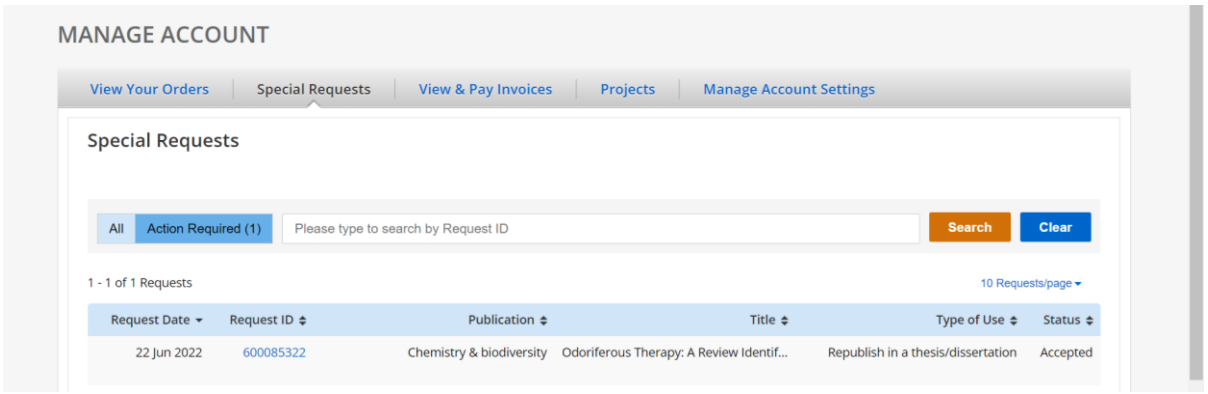
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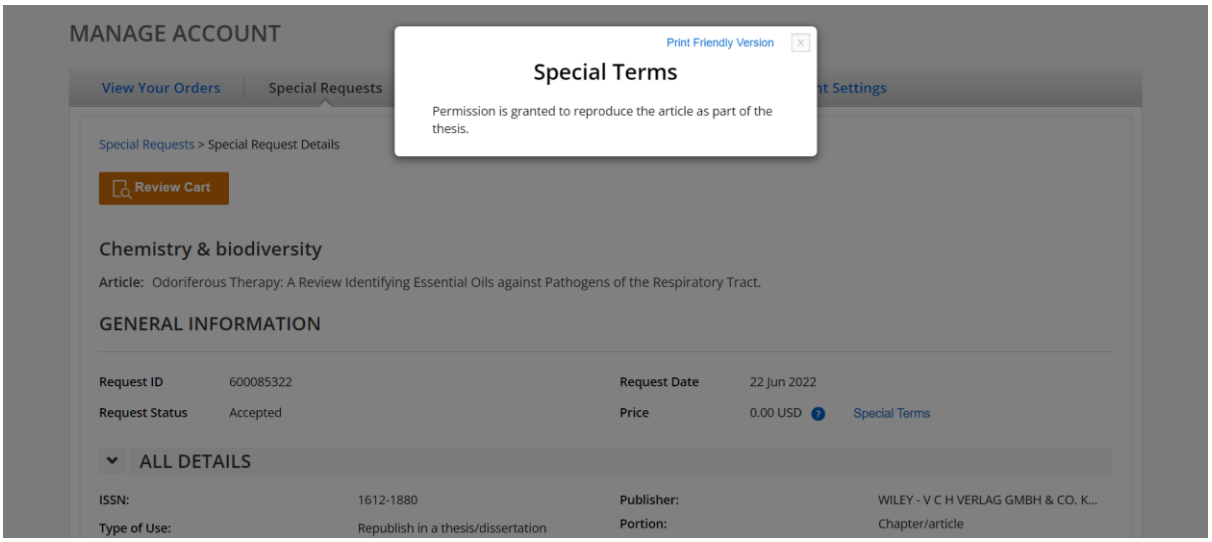
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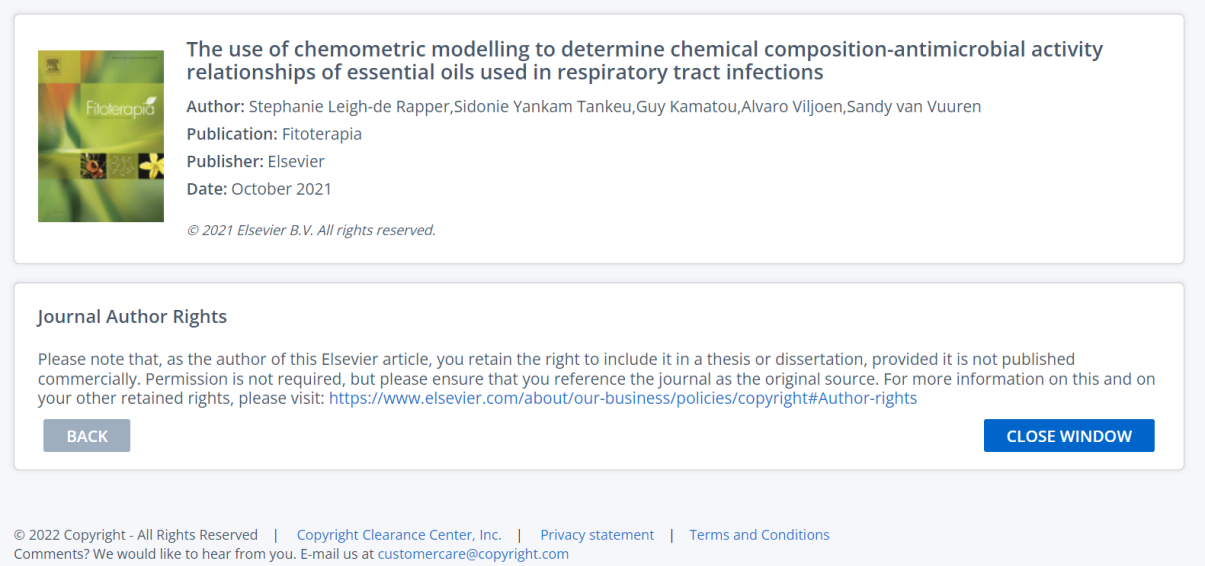
de Rapper, S. and van Vuuren, S.F. (2020). Odoriferous Therapy: Identifying the Antimicrobial Potential of Essential Oils against Pathogens of the Respiratory Tract. *Chemistry & Biodiversity*. 17. doi: 10.1002/cbdv.202000062.





Article 2

Leigh-de Rapper, S., Tankeu, S.Y., Kamatou, G., Viljoen, A. and van Vuuren, S.F. (2021) The use of chemometric modelling to determine chemical composition-antimicrobial activity relationships of essential oils used in respiratory tract infections. *Fitoterapia*. doi: 10.1016/j.fitote.2021.105024.



The use of chemometric modelling to determine chemical composition-antimicrobial activity relationships of essential oils used in respiratory tract infections

Author: Stephanie Leigh-de Rapper,Sidonie Yankam Tankeu,Guy Kamatou,Alvaro Viljoen,Sandy van Vuuren
Publication: Fitoterapia
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Leigh-de Rapper, S., Viljoen, A.M. and van Vuuren, S.F. (2021) Essential Oil Blends: The Potential of Combined Use for Respiratory Tract Infections. *Antibiotics*. 10(12): 1517. doi: 10.3390/antibiotics10121517

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Article 4

Leigh-de Rapper, S., Viljoen, A.M. and van Vuuren, S.F. (2022) Optimizing the antimicrobial synergism of *Melaleuca alternifolia* (tea tree) essential oil combinations for application against respiratory related pathogens. *Planta Medica*, 2022.

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Appendix C – Ethics Waiver

Human Research Ethics Committee (Medical)

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Medical School Secretariat: Tobias Health Sciences Building, 2rd floor Tel +27 (0)11-717-2700
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Ref: W-CJ-160720-2 20/07/2016

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, S de Rapper (Student no 0704317T).**

Project title: The prediction of interactive efficacies in essential oils used for respiratory infections.

Reason: This is a laboratory study using bacterial cultures. The bacterial strains include:
Staphylococcus aureus (ATCC 25924), *Streptococcus agalactiae* (ATCC 55618), *Streptococcus pneumoniae* (ATCC 49619), *Streptococcus pyogenes* (ATCC 12344), *Pseudomonas aeruginosa* (ATCC 27858), *Escherichia coli* (ATCC 25922), *Haemophillus influenzae* (ATCC 19418), *Klebsiella pneumoniae* (ATCC 13883), *Moraxella catarrhalis* (ATCC 23246), *Morganella morganii* (ATCC 25829), *Mycobacterium smegmatis* (ATCC 14468), *Candida albicans* (ATCC 10231) and *Cryptococcus neoformans* (ATCC 14116).

There are no human participants.

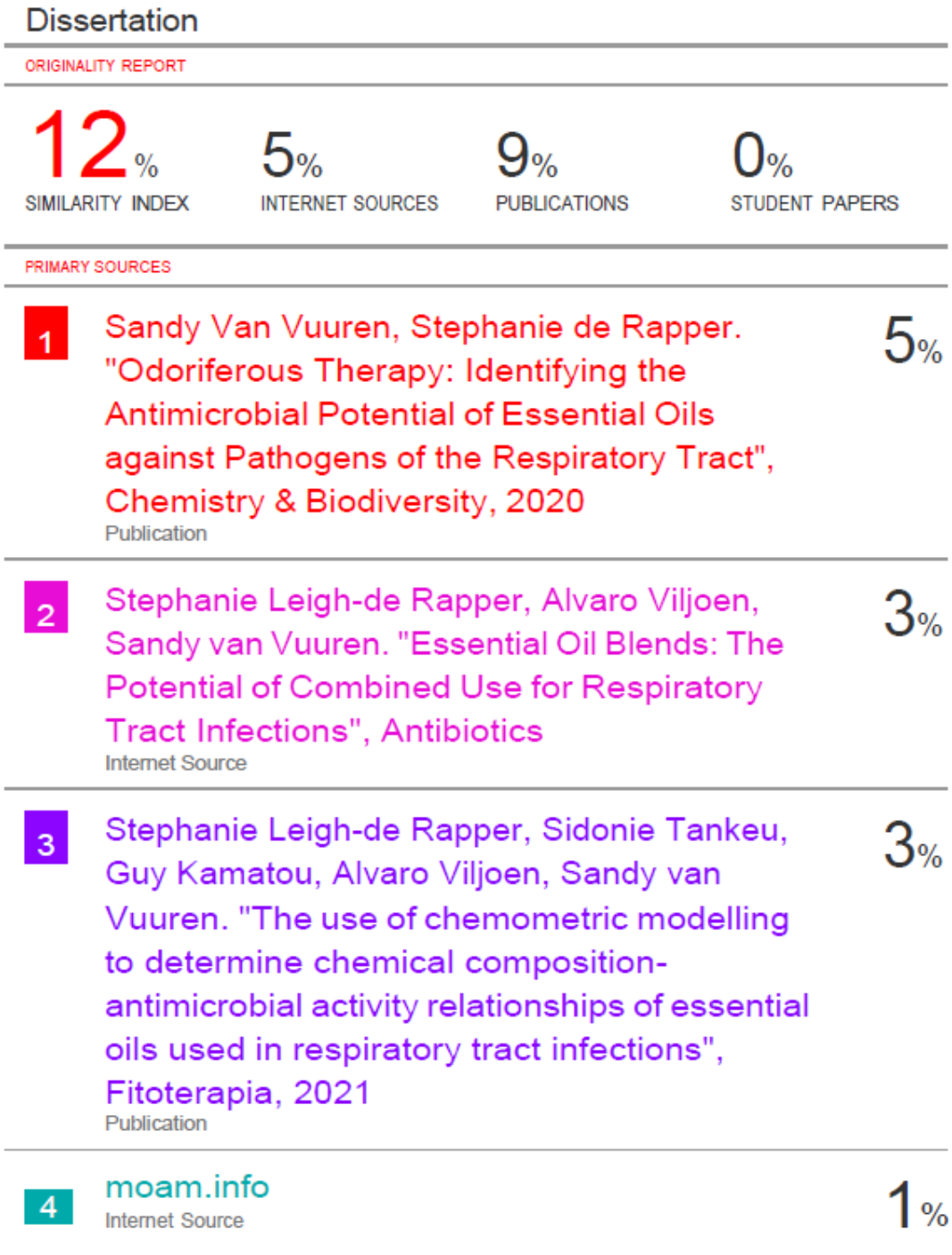
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Appendix D – Plagiarism Report



Dissertation

ORIGINALITY REPORT

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SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

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1	Sandy Van Vuuren, Stephanie de Rapper. "Odoriferous Therapy: Identifying the Antimicrobial Potential of Essential Oils against Pathogens of the Respiratory Tract", Chemistry & Biodiversity, 2020 Publication	5%
2	Stephanie Leigh-de Rapper, Alvaro Viljoen, Sandy van Vuuren. "Essential Oil Blends: The Potential of Combined Use for Respiratory Tract Infections", Antibiotics Internet Source	3%
3	Stephanie Leigh-de Rapper, Sidonie Tankeu, Guy Kamatou, Alvaro Viljoen, Sandy van Vuuren. "The use of chemometric modelling to determine chemical composition-antimicrobial activity relationships of essential oils used in respiratory tract infections", Fitoterapia, 2021 Publication	3%
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