DURBAN UNIVERSITY OF TECHNOLOGY
INYUVESI YASETHEKWINI YEZOBUCHWEPHESHE

Formulation and *in vitro* analysis of essential oil blended (*Cymbopogon citratus, Syzgium aromaticum and Melaleuca alternifolia)* biocompatible hand sanitizer against common bacterial pathogens.

CINDY SAUNDERS

Submitted in fulfilment of the requirements of the degree of Master of Somatology: Faculty of Health Sciences at the Durban University of Technology, Durban, South Africa.

.

FINAL COPY APPROVED FOR SUBMISSION

11-04-2024

Supervisor: **Date**

Dr. A. K. Puri (Ph. D)

11 /04/2024

Co Supervisor: **Date**

D. Borg

DECLARATION

I hereby declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Health Science in Somatology, to the Durban University of Technology Durban, South Africa. It has not been submitted before for any degree or dissertation to any other institution.

_____

Cindy Saunders
11-04-2024

CONTENTS

ACKNOWLEDGEMENTS

- **Durban University of Technology and Biotechnology Department –** for allowing me to conduct research in your laboratory.
- **Dr Adarsh Kumar Puri –** My principal supervisor, for enlightening me with the passion of research. Your enthusiasm and endless hours of discussion and education will forever be with me.
- **Andrew Saunders –** My husband, for the endless support and encouragement in times of despair and for being the best dad to our son Daniel.
- **Daniel Saunders –** My son, the light at the end of a dark day, always bringing a smile to my face and reminding me of the small things in life.
- **The Saunders family** – my in-laws Stephen and Janine Saunders, brother-in-law Michael saunders and sister-in-law Megan Bahlmann for providing strength and support.
- **The Pieterse family –** My mom and dad, Juanita and Wilhelm Pieterse for providing me with the best life has to offer, I appreciate all you have contributed to my life for making me the woman I am today. Supporting my unique decisions in life**.**
- **Mrs Dorinda Borg** – my co-supervisor and mentor for your support and encouragement. Your passion for education and research planted the seed for my education.
- **Ms. Nomakhosi Mpofana** – my mentor and colleague – for the moral support and steering me on the path towards my goal.
- **Stellenbosch University –** for the GC/MS testing and results of essential oils.
- **Department of Somatology –** for your support.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

GRAS – Generally Regarded as Safe

MIC – Minimum inhibitory concentration

ºC – Degrees Celsius

PSI – Pounds per square inch

h – hour

L – Litre

RPM - Revolutions Per Minute

GC-MS – Gas chromatography – mass spectrometry

FTIR – Fourier transform infrared.

RSM – Response surface methodology

ATCC – American Type Culture Collection

EPI- Efflux Pump Inhibitors

VOC – Volatile Organic Compounds

EO – Essential oil

LG - Lemongrass

TT – Tea tree

C – Clove

RT – Retention time

V/V – Volume / Volume

ABSTRACT

Essential oils (EOs) are predominantly known for their use in aromatherapy, cosmetic, food and pharmaceutical industries. Combination of EOs may result in holistic synergistic effects due to a blended biocompatible mixture. In this study, three EOs were selected out of the seven EOs tested for their efficacy against Gram-positive and Gram-negative bacteria based on the results of disc diffusion. The Minimum Bactericidal Concentration (MBC) of EOs from *Cymbopogon citratus* (lemongrass), *Melaleuca alternifolia* (tea tree) and *Syzgium aromaticum* (clove) were determined against *Micrococcus luteus*, *Bacillus subtilis*, *Bacillus cereus*, *Klebsiella pneumoniae* and *Escherichia coli* ATCC 11775. The EOs were analysed using gas chromatography mass spectrometry (GC-MS) to identify the volatile organic compounds. Geranial, neral, neryl acetate and β-caryophyllene were present in highest concentrations among 48 chemicals identified in lemon grass essential oil (LGEO). GC-MS identified 17 chemical compounds in clove EO (CEO) with eugenol, eugenol acetate and β-caryophyllene present in highest concentrations. A total of 52 compounds were identified with terpineol-4, γ-terpinene, α-terpipene and 1,8-cineole (eucalyptol) being the most prevalent compounds in tea tree EO (TTEO). Response surface methodology (RSM) using central composite design (CCD) was used to develop a mathematical model that determined the optimal concentrations of LGEO, TTEO and CEO as 0.10%, 0.11% and 0.10%, respectively. This investigation highlighted the importance of microbiological techniques and statistical optimization tools to study the synergistic effect of selected EOs towards developing a combined EO - based antibacterial treatment technology. Overall, this study showed the potential of developing a biocompatible hand sanitizer which resulted in marked log reduction of bacteria. Further study on the efficacy of the developed EO-mixture against viruses, fungi and nematodes will provide useful scientific knowledge.

CHAPTER 1: INTRODUCTION

1.1 INTRODUCTION

Proper hand hygiene is the most effective method for the prevention of infection and cross-contamination of pathogens (Vermeil 2019). By preventing the spread of bacterial pathogens, we can reduce the number of infections that are potentially treated using antibiotics. This provides less opportunity for bacteria to develop and spread as drug-resistant strain (Ponen 2020). Products used to inhibit the growth of bacteria usually contain harsh chemicals that are unfavourable for our skin therefore the focus to create a product that can inhibit the growth of bacteria without damaging the integrity of the skin is needed. This chapter includes background, aims and objectives of the study as well as the layout for the rest of the research.

1.2 BACKGROUND OF THE STUDY

The World Health Organisation (2019) declared a global pandemic of Covid-19 in 2020. A great emphasis was put on hand hygiene to prevent the spread of the virus. The awareness, use and demand for hand sanitizers has seen exponential growth during the global Covid-19 pandemic. More specifically, a 70% alcohol-based hand sanitizer was recommended by the World Health Organisation (2019). The high demand for the alcohol-based hand sanitizer resulted in a limited supply of isopropanol and ethanol (Booq *et al.* 2021).

The application of proper hand hygiene can also be applied to prevent the spread of bacterial infection. Bacterial infections can be as minor as the common cold to severe life-threatening infections or even death. A global total of 827 000 deaths are reported per annum because of a lack of sanitation and water available for the purpose of hygiene (Anisha Bhatia 2021).

Unicef (2020) substantiates that 18 million people or 47 per cent of South Africans are without basic hand washing facilities at their residence. In the event of a lack of water for hand washing, a hand sanitizer may be used. However, the frequent use of highly concentrated alcohol-based hand sanitizers has a negative effect on the environment as well as the health and well-being of the user.

Ethanol and chemical detergents are not easily degradable, which remains in the environment for a long period of time therefore contributing to environmental pollution (Daverey and Dutta 2021). High levels of alcohol-based hand sanitizers may compromise the function of the epidermis and lead to cutaneous irritations such as angioedema, dermatitis, eczema, and dryness of the hands (E.G Hecht 2020). Additionally, these often result in undesirable side effects such as swelling of the skin, itchiness, redness, scaling, thickening and cracks of the skin and may even lead to bleeding (Chakraborty *et al.* 2021).

In some faiths, such as Islam and certain branches of Buddhism, the use of alcohol is generally not promoted (Shiraly *et al.* 2021). However, people conflicted using alcohol in any form are also expected to adhere to the current covid-19 pandemic gazetted health protocol which stipulates the use of a 70% alcohol-based hand sanitizer.

Therefore, a natural aromatherapy-based hand sanitizer may be a promising option due to the chemical constituency of the selected blend of essential oils that are known for their antimicrobial, anti-inflammatory, and healing properties (Unalan and Boccaccini 2021).

1.3 AIM OF THIS STUDY

The aim of the study is to test the antibacterial efficacy of essential oils for the formulation of a biocompatible blended hand sanitizer.

1.4 RESEARCH OBJECTIVES

1.4.1 **Objective 1**: To investigate the composition of lemongrass, clove and tea tree essential oils by Gas Chromatography-Mass Spectroscopy (GC-MS).

1.4.2 **Objective 2**: To determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of these oils against most common bacterial pathogens.

1.4.3 **Objective 3**: To statistically optimize the most significant variables using Response Surface Methodology (RSM) to achieve maximum efficacy and its validation.

CHAPTER 2: REVIEW OF LITERATURE

2.1 ESSENTIAL OILS

Essential oils are highly concentrated, volatile aromatic extracts of plant material. These intricate compounds that may comprise of over 300 compounds (Conde-Hernández *et al.* 2017). Plants generate essential oils as secondary metabolites, useful to protect plants against bacteria, fungi, insects and larger animals (Herman *et al.* 2019).

The diversity of essential oils has been demonstrated globally through many cultures for many centuries. The use of these plant materials have evolved since it was first recorded in 4500BC by the ancient Egyptians for ointments, cosmetics and perfume (Elshafie and Camele 2017). Ancient Egyptians would also use essential oils for the mummification process, these oils have recently been studied for their antibacterial properties (Barnes, Whiffin and Bulling 2019).

Today, essential oils are analysed for chemical compounds and individually studied for significant medical conditions such as cytotoxicity against a variety of cancer cells (Trang *et al.* 2020).

2.1.1 EXTRACTION OF ESSENTIAL OILS

Extraction is the process to obtain a substance from somewhere else, either by chemical or industrial process (Collins Dictionaries 2018). The concentrated essential oil is extracted from different materials of the plant, either the entire plant or different segments such as the seeds, roots, wood, flower, peel, herbs, twigs, bark, rhizomes or buds (Wania 2021). Different extraction process methods are used to extract the essential oil from the type of medium it is found in. Distillation is the most used method to extract essential oil. Different extraction methods contribute to the type of chemical constituents that are found in the essential oils.

2.1.2 CHEMICAL COMPOSITION OF ESSENTIAL OILS

The volatile essential oils comprise of complex secondary metabolites linked to the communication and defence mechanism of the plant. The classification of these chemical

constituents include: unsaturated and saturated hydrocarbons, ketones, esters, ethers, oxides, alcohols, terpenes and aldehydes (da Silva *et al.* 2022). Table 2.1 provides examples of essential oils and their constituents.

Table: 2.1 Examples of essential oils and their constituents

Essential oil constituent classification	Example of essential oil constituent	Example of essential oil containing constituent	Activity	Reference
Hydrocarbon	Phellandrene, dodecane	Nutmeg leaf oil, cinnamon, pine, ginger	Antimicrobial, increase energy and reduce pain	(Nazir and Gangoo 2022) (Kapelle, Souhoka and Walla)
Ketones	Thujone, pulegone, camphor and verbenone, Dill, Hyssop, Rosemary, Spearmint.	Thuja, sage, hyssop, rosemary and red cedar	Cell regenerations, antiviral, mucolytic, and digestive.	(Herman *et al.* 2019)
Esters	Eugenol, geranyl, acetate and linalyl acetate.	Clary sage, bergamot, petit grain, sweet marjoram, benzoin, Roman Chamomile, wintergreen and Jasmine.	Relaxing, Calming, antispasmodic, sedative anti-inflammatory, analgesic and antifungal.	(Nazir and Gangoo 2022) (Herman *et al.* 2019)
Ethers	Eugenol, methyl chavicol and anethol	Clove, *L. rugosa*	Antibacterial, antifungal, antiviral, anticancer activity, antiparasitic,	(Sabaoui and Lakhdar 2021) (Van *et al.* 2021)

			analgesic, anti-inflammatory, antioxidant and anesthetic.	
Oxides	1,8-cineole also called eucalyptol.	Eucalyptus, niaouli and rosemary.	Expectorants, antimicrobial, antivral and anti-inflamatory	(Ak Sakallı *et al.* 2022)
Monoterpenes	Camphene, carene, cymene, limonene, myrcene, phellandrene, pinene, sabinene and terpinene.	Black pepper essential oil, grapefruit, lemon and lime,	Antibacterial, antiviral, antiseptic, decongestant, anti-inflammatory and analgesic.	(Spesyvyi, Španěl and Sovová 2019) (Kim *et al.* 2020)
Alcohols	terpinen-4-ol, geraniol, citronellol, menthol, terpineol,	tea tree essential oil,	Antibacterial, antibiofilm, antifungal	(Cordeiro *et al.* 2020)
Phenols	Thymol, carvacrol, eugenol	Cinnamon leaf, clove bud, oregano and fennel	Antimicrobial, antibacterial, antifungal	(Saroj *et al.* 2020)
Sesquiterpenes	Caryophyllene, copaene and panasinsen	German chamomile, yarrow and arnica	Anti-inflammatory, antiviral, mucolytic and immune stimulating	(Ogundajo *et al.* 2021) (Herman *et al.* 2019)
Aldehydes	Cinnamaldehyde, citral, geranial, citronellal and neral	Cinnamon, lemongrass, cilantro and Melissa oil	Antimicrobial, anti-inflammatory and antioxidative	(Aljaafari *et al.* 2022)

2.1.3 MULTIFARIOUS USES OF ESSENTIAL OILS

EOs have been known for multifarious uses. Fig 2.1 below highlights the wide variety of sectors. Each topic will be discussed below.

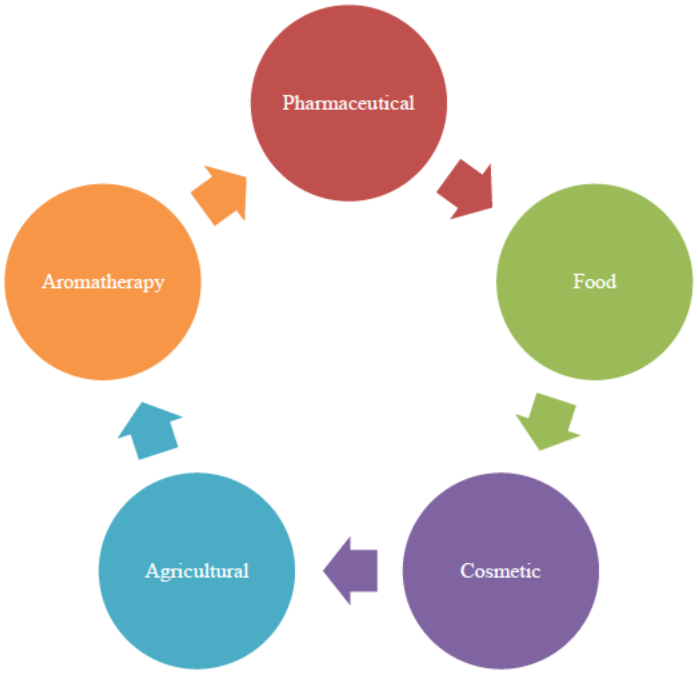


Fig 2.1 Multifarious uses of essential oils

2.1.3.1 PHARMACEUTICAL

Essential oils have been used to treat many health ailments based on their biological activities (Soares *et al.* 2021). Some pharmaceutical uses of essential oils include antimicrobial, wound-healing, anti-inflammatory, antioxidant and anxiolytic (Cimino *et al.* 2021). Recently, essential oils are being used to complement the pharmaceutical industry as either an additive, synergistic or antagonistic (Aljaafari *et al.* 2021). The use of antimicrobial agents and essential oils may have a multi-target approach to prevent drug resistant pathogens and enhance the activity of the antimicrobial agent (Ju *et al.* 2022).

2.1.3.2 NEUTRACEUTICAL

Food contamination has a negative effect on the food industry and human health. This results in the need for preservatives, however, synthetic preservatives have a negative effect on the environment and the health of humans (Angane *et al.* 2022). Antimicrobials that are plant-based such as essential oils are gaining more interest as a synthetic alternative as they are generally recognised as safe (GRAS) and eco-friendly in nature (Maurya *et al.* 2021). Recently a study performed by Galovičová *et al.* (2021) on the antimicrobial effects of *Thymus vulgaris* proved to be effective and could be used as a natural supplement in the food industry to extend the shelf life of food.

2.1.3.3 COSMETIC

A large portion of cosmetic products contains fragrance. However, artificial fragrance has a negative health risk and the demands from the international regulations has therefore promoted the interest for a natural, plant-based ingredients including essential oils in the cosmetic industry (Sharmeen *et al.* 2021). This has promoted more research in essential oils and their individual components. Essential oils are now used as active ingredients as well as natural preservatives in a variety of cosmetic products (Guzmán and Lucia 2021). A recent study in Indonesia on four essential oils (Clove, Nutmeg, patchouli and citronella) was performed by Rahmi *et al.* (2021) to determine the antiaging potential based on their antioxidant activities. Antiaging and the improvement of wrinkles were proven, however high concentrations resulted in the potential for skin irritations.

2.1.3.4 AGRICULTURAL BIOSTIMULATION

Godlewska, Ronga and Michalak (2021) emphasised the need to develop environmentally-friendly methods to protect crop and increase plant growth. The multi-compounds in essential oils are good bio stimulants that can be used as an organic pesticide, fungicide, bactericide, or weedicide which decreases attacks on crops, and prevents the spoilage of food (Yasin *et al.* 2021). Recently Aimad *et al.* (2021) studied the antibacterial, antioxidant, insecticidal properties and chemical

composition of *Mentha. pulegium (*Labiatae) essential oil. The study revealed that *M. pulegium* L. was active against the tested insect, pest and microbes while its antioxidant property proven to be a natural drug to aid in food crop production.

2.1.3.5 AROMATHERAPY

Aromatherapy is an alternative complementary therapy whereby essential oils enter the body through inhalation, bathing in essential oil infused water or massage (Her and Cho 2021). The diverse advantages of the use of aromatherapy include the improvement of mood, depression, anxiety and stress, as well as improve immune function which has a general improvement on the body's health and wellbeing (Aćimović 2021). There are many studies highlighting the complementary benefits of aromatherapy. Some of the studies of aromatherapy include the study by Tang *et al.* (2021) on the significant improvement of the quality of sleep.

2.1.4 TEA TREE ESSENTIAL OIL

Melaleuca alternifolia, commonly known as tea tree (TT), is a member of the Myrtaceae family. Grown along the coastal area, it is native to Australia (Li *et al.* 2021). The essential oil produced from the leaves of *Melaleuca alternifolia* is known as TTEO. Annually the globe produces 720 tonnes of TTEO, the major states producing majority of the oil includes Queensland and New South Wales in Australia (Businesswire 2019). TTEO has been used in the topical application of acne, burns, arthritis, tinea, vaginal thrush, and dandruff due to its anti-inflammatory, antioxidant, anti-bacterial, anti-viral, and anti-fungal properties (Taalab *et al.* 2021; Mori, Natsuki and Toyonobu 2022) identified major components using GC-MS as α-terpinene (alpha-pinene), γ-terpinene (gama-terpinene), terpinene-4-ol, and eucalyptol (1,8-cineole) as shown in Fig.2.2 below.

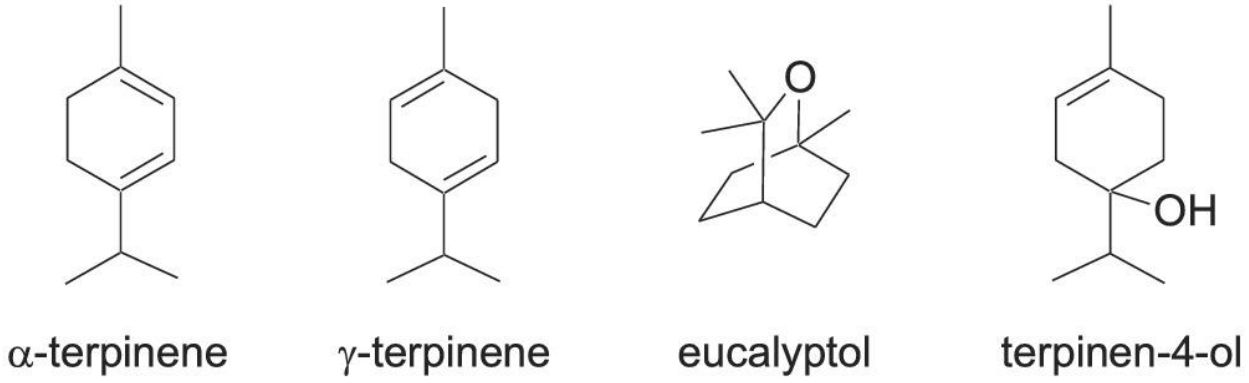


Fig. 2.2 Major components identified in tea tree essential oil.

TTEO shows broad spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria. Hydrocarbons penetrate the biological membrane of the bacteria. Lysis leads to the inhibition of respiration and leakage of irons resulting in the disruption of the essential function of the bacteria (Muta *et al.* 2020). Recently scientific studies showed positive results *in vitro* and *in vivo* in the treatment of human breast (MCF-7) and prostate (LNCaP) cancer (Clark *et al.* 2021).

2.1.5 CLOVE BUD ESSENTIAL OIL

Syzgium aromaticum, the dry flower bud known as clove (C), is a member of the Myrtaceae family, indigenous to the Indonesia Maluku islands (Hassine *et al.* 2021). Indonesia produces around 80% of the 6 000 tonnes of CEO produced annually (Danthu *et al.* 2021). CEO is used in the medicinal, pharmaceutical, agricultural and cosmetic industries for its antioxidant, antimicrobial, antipyretic, analgesic and anti-inflammatory properties (Banerjee *et al.* 2020). Chen *et al.* (2022) identified the following major components of clove bud using GC-MS: eugenol (43.51%), β-caryophellene (36.34%), eugenyl acetate (8.73%), and α-humulene (4.92%). Chemical structure can be seen below in fig 2.3 (Haro-González *et al.* 2021). Recently CEO was used in a scientific study by Michalczyk and Ostrowska (2021), to prove its antifungal properties in humans and animals against fungal dermatosis.

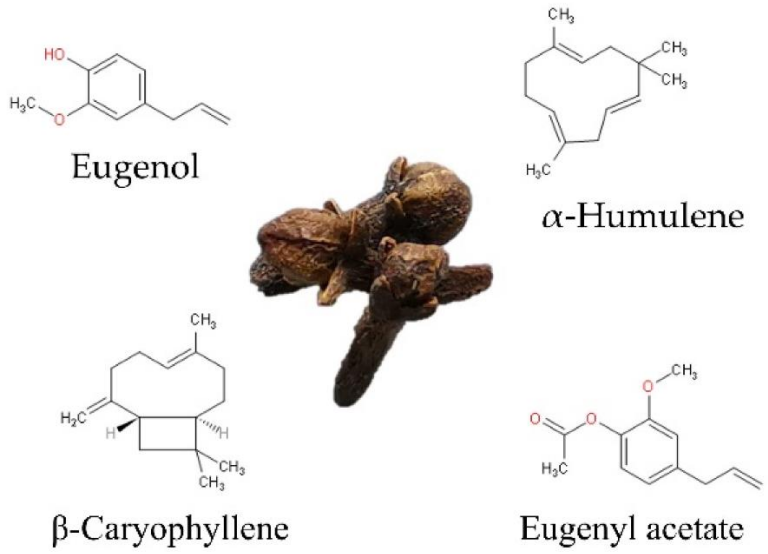


Fig. 2.3 Major components identified in clove bud essential oil

2.1.6 LEMONGRASS ESSENTIAL OIL

Cymbopogon citratus commonly known as Lemongrass (LG), is a member of the Gramineae family (Hartatie *et al.* 2019). Native to India and Sri Lanka, LG is a perennial grass grown in the tropical climates (Singh *et al.* 2018). Globally, 1000 tonnes of LGEO produced each year, with the major exporter being Cochin Port in India (Mukarram *et al.* 2021). LGEO is known for its wide application of uses in the food technology, cosmetic and medicinal sectors due to its analgesic, insecticidal, antimicrobial, antidiarrheal, antiamoebic, antiseptic, antitussive, antioxidant, anti-inflammatory and anticancer activities (Mukarram et al., 2021). Mahmoud *et al.* (2022) identified 31 chemical compounds using GC-MS analysis with two major compounds being geranial and neral which can be seen below in fig 2.4 (Barros *et al.* 2020).

Johnson *et al.* (2021) performed a recent study whereby LGEO was used in conjunction with TTEO to enhance the effectiveness of chlorhexidine on reducing *Candida auris* colonies.

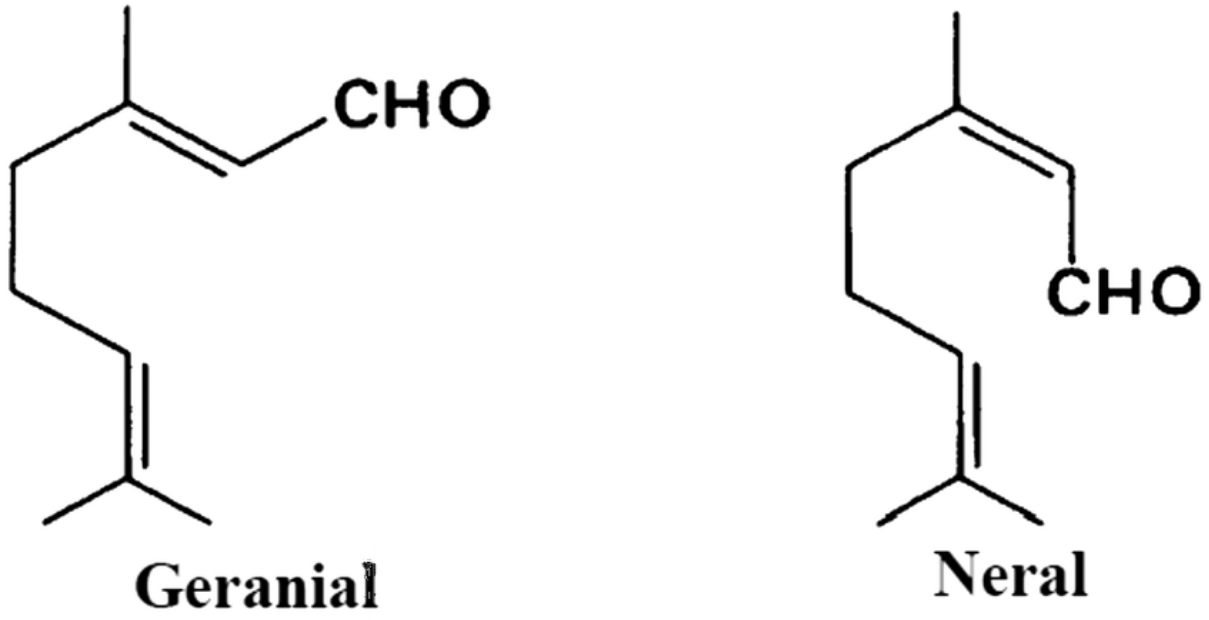


Fig. 2.4 Major compounds identified in lemongrass essential oil

2.2 THE SKIN ANATOMY AND PHYSIOLOGY

The skin, the largest organ of the human body, weighing approximately 15% of the total body mass, is comprised of three main layers: The epidermis, dermis, and subcutaneous fat layer (Lai-Cheong and McGrath 2017; Dehdashtian *et al.* 2018; Hoffman 2021). The main function of the skin is to protect the body against UV light, chemicals, injury, and pathogens by means of a barrier (Hani Yousef; Mandy Alhajj; Sandeep Sharma 2021).

2.3 THE NEED FOR A BIOCOMPATIBLE HAND SANITIZER

Proper hand hygiene is the most effective method for the prevention of infection and cross-contamination of pathogens (Vermeil, 2019). The ingredients of these products have a negative effect on humans and the environment. The compounds found in hand sanitizers and hand hygiene products contain chemical surfactants that are synthesized from petrochemicals which are not easily degradable (Daverey and Dutta, 2021).

2.3.1 ENVIRONMENTAL IMPACT

Synthetic chemical detergents from hand sanitizers are emerging contaminants that are toxic to many living organisms. This effects different ecosystems: soil, water, and sediments. The increase in the number of hydrocarbons in the environment due to the petrochemicals create unfavourable conditions in the aquatic environment as well as creating antibiotic resistance in micro-organisms. This may result in polluted water being untreatable. These problems include the inhibition of bio degeneration of organic compounds, interruption of oxygen diffusion as well as foaming.

2.3.2 HUMAN IMPACT

Overuse of hand sanitizers has a negative effect on human health. The increase in hand washing using detergent and disinfectants can lead to the irritation of the hydrolipid barrier of the skin, which may also lead to contact dermatitis. (Darlenski and Tsankov, 2020). Ethanol has been reported by Malabadi *et al.* (2021) to decrease the barrier function on the skin. Thus, causing chronic irritation while allowing the skin to be more susceptible to harmful pathogens.

2.4. METHODS OF ESTIMATION OF ESSENTIAL OILS

2.4.1 NUCLEAR MAGNETIC RESONANCE (NMR)

Nuclear Magnetic Resonance (NMR) is the most significant analytical spectroscopic method for the study of molecules by using the electromagnetic radiations in a strong magnetic field (Zia *et al.* 2019). NMR is one of the most convincing methods to detect and confirm different compounds present in essential oil. Recently, Freitas *et al.* (2018) used NMR spectroscopy to investigate essential oils from different species of *Ocimum*. The authors showed that NMR was a better method to detect eugenol, estragole, methyl cinnamate and eucalyptol present in essential oils from different species of *Ocimum*. Previously, Salvino *et al.* (2022) , Fekri *et al.* (2021) and Mahanta *et al.* (2021) have used NMR to identify essential oil components in bergamot, geranium and black turmeric, respectively. A combination of GC-MS and NMR methods were used to detect

biomolecules in vetiver, lavender, and rosemary essential oil (Halahlah *et al.* 2021; Wang *et al.* 2021; Schripsema, da Silva and Dagnino 2022).

2.4.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC is a high-pressured analytic method used to separate, identify, and quantify either a complex biological sample or synthetic chemicals (Aryal 2022). HPLC is the preferred method to use when analysing fewer volatile constituents of essential oils (Sharmeen et al., 2021). Recently Purba and Paengkoum (2019) used reversed-phase high-performance liquid chromatography in conjunction with diode-array detection (HPLC-DAD) to estimate and evaluate eugenol, ascorbic acids, flavonoids and phenolic acids in the essential oil of *Piper betle.* HPLC and GC-MS are frequently used together to evaluate chemical compositions in essential oils. Recently, Asteggiano *et al.* (2021) used HPLC to analyse acidic triterpenes while GC-MS was used for the analyses of volatile terpenes in essential oil from *Protium hetaphyllum.*

2.4.3 INFRARED SPECTROSCOPY

2.4.3.1 NEAR-INFRARED REFLECTANCE SPECTROSCOPY (NIRS)

NIRS is a rapid, chemical-free, non-destructive analytic method for the evaluation of chemical constituents in essential oils (Basyir, Munawar and Aisyah 2021). Light irradiates through the sample creating a vibration of the chemical bonds (O-H, N-H and C-H groups) within the molecules which translates into an electromagnetic wave creating the NIRS (Yu 2021). The use of NIRS has recently been used to efficiently identify phytosterols, rhodinol quality, impurities and quality of the oil in vegetable oil, citronella essential oil, and patchouli oil (Liu *et al.* 2019; Meilina and Munawar 2021; Wahyudi *et al.* 2022). It is common for NIRS to be used in conjunction with GC-MS as seen in the studies by Ercioglu, Velioglu and Boyaci (2018) and Wang *et al.* (2018) whereby terpenoid compounds and antioxidants were investigated in aromatic plants (Rosemary,

Basil, Juniper, Black pepper, Laurel, Lavender, Spearmint and Ginger) and *Cinnamomum longepaniculatum* respectively.

2.4.3.2 FOURIER TRANSFORM INFRARED (FTIR)

Fourier transform infrared spectroscopy is a highly sensitive, non-destructive, well-established vibrational technique used to obtain an infrared spectrum of the emission or absorption of a solid, liquid, or gas at a molecular level (Petit and Puskar, 2018). FTIR is difficult to interpret and may require complementary characterization techniques to avoid unambiguous interpretation. This method of analyses is not element specific. IR-active vibrational modes of different chemical bonds may have the same frequencies. FTIR is commonly used in the study of essential oils to test for adulteration within the essential oil as seen in the studies by Fahmi et al. (2020) and Cebi et al. (2020) in the study of patchouli essential oil and lemon essential oil respectively. FTIR was also used to test the quality of over 30 essential oils in a study by Agatonovic-Kustrin et al. (2020).

2.4.4 GAS CHROMATOGRAPHY (GC)

Gas Chromatography (GC) is a sensitive, widely used, effective analytical technique for the separation of chemical compounds of a complex sample based on the polarity of the compound (Mukadam et al.). GC can be used to analyse solids, liquids and gases. Solids and liquids need to be dissolved in volatile solvent prior to being analysed. The volatilized sample is transported during the mobile phase through the column by the carrier gas. The courier gas is generally an unreactive or inert gas such as nitrogen, hydrogen, argon, or helium. This technique is the chosen method to analyse essential oils as the components of essential oils are mainly volatile or semi-volatile (Cagliero et al., 2022, Mukadam et al.). Different types of detectors may be used based on the properties of analyte:

2.4.4.1 FLAME IONIZATION DETECTOR (FID)

Flame Ionization Detector is a mass sensitive detector for the analysis of chemical components of a gas stream using GC. FID uses a flame to ionize the organic compounds that contain carbon molecules (Rasmussen and Rosenfjeld, 2020). Studies that have adopted GC-FID to analyse the organic compounds of essential oils include the study of the plants *Mentha citrate (*Ouakouak et al., 2019*), Citrus aurantifolia (*Lemes et al., 2018*)* and *Origanum Vulgare* L. (Khan et al., 2019).

2.4.4.2 THERMAL CONDUCTIVITY DETECTOR (TCD)

Thermal Conductivity Detector (TCD) is a non-destructive, chemical detector used in gas chromatography. This method of detection measures specific chemicals based on the conductivity of analyte and the courier gas, therefore not widely used (Mukadam et al.). Cagliero et al. (2018) used GC in conjunction with TCD and FID to analyse the aqueous samples in the fragrance samples of essential oils. Zhang et al. (2019) also used TCD in the study of antibacterial activities of essential oils, however this study used the TCD to measure the volatile metabolites in the headspace of a vial containing bacterial broth. Carbon dioxide produced by the bacteria was measured to analyse the antimicrobial activity of *Warburgia ugandensis* and *Lonicera haponica*.

2.4.5 MASS SPECTROMETRY (MS)

Mass Spectrometry (MS) is the leading technique used in the identification, analyses, and quantification of the structures of a molecule (Bano et al., 2021). MS measures the molecular weight as well as the positive ions that are formed during the ionization process (Noriega et al., 2021). Essential oils consist of a mixture of complex components and are usually chemotyped by GC and MS analyses (Rojas *et al.* 2021). MS, in conjunction with GC (GC-MS), yields more accurate results with higher sensitivity and a better detection limit. Similarly, LC-MS (Liquid Chromatography-Mass Spectrometry) can be used for the precise phytochemical detection and evaluation of several essential oils.

Table 2.2 Essential oils analysed for their volatile constituents.

Method	Components measure	Essential oil	Reference
GC-MS	38 essential oils were identified with the largest component being citronellol (34%).	*Rosa damascena* essential oil	(Cebi et al., 2021)
GC-MS	5 major components identified with Eugenol (53.23%) being the largest component.	*Syzygium aromaticum*	(Teles et al., 2021)
GC-MS	53 components identified with α-pinene (21.09%) being the largest component.	*Syzygium cumini* (Pomposia)	(El-Nashar et al., 2021)

2.5 ANTIMICROBIAL ACTIVITIES

Essential oils are well-known for their antimicrobial activities depending on their chemical composition. Biomolecules present in essential oils may inactivate or inhibit the growth of microorganisms depending on the mechanism of action (Aljaafari et al., 2021). The antibacterial mechanism of action will depend on the essential oil used or the strain of bacteria.

Gram-negative bacteria have a large lipopolysaccharide that are attached to the outer membrane of the cell, which is absent in Gram-positive bacteria (as seen in Fig 2.5). Essential oils act by inactivating the outer cell wall of bacteria or deactivating the membrane proteins efflux system therefore allowing essential oils to enter more easily (Nair *et al.* 2022).

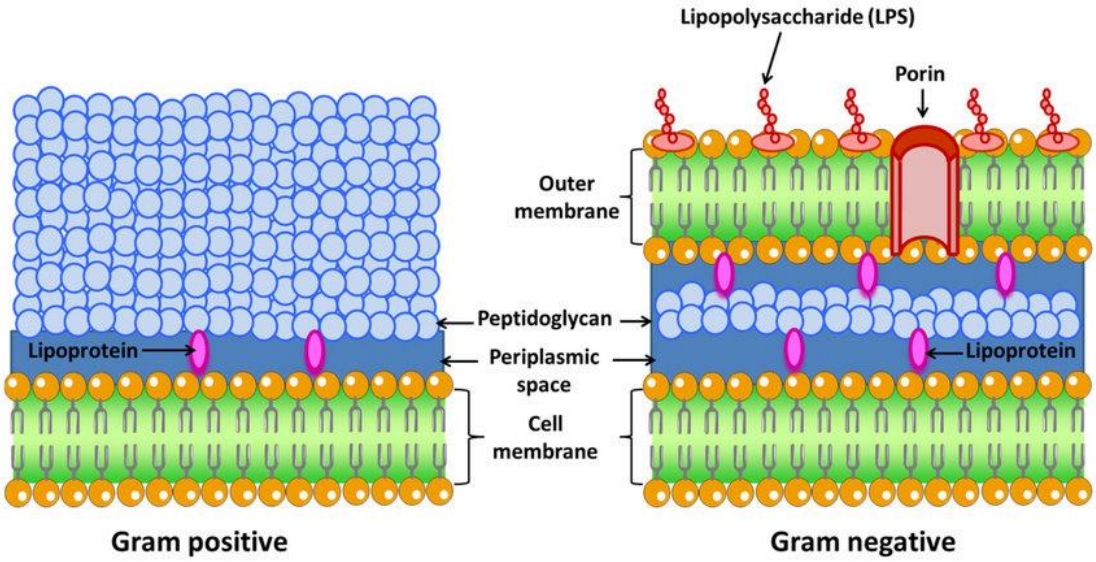


Figure 2.5 Cell walls of gram positive and negative bacteria

2.5.1 MEMBRANE DISRUPTION

The bacterial membrane is responsible for the regulation of the osmotic pressure within the cell (Yang et al., 2021). Antimicrobial effects of essential oils target both Gram-positive and Gram-negative cell walls (Yap et al., 2021). Once the cell wall has been compromised, intracellular leakage occurs leading to the death of the cell.

2.5.2 EFFLUX PUMP INHIBITION

The bacterial membrane has an efflux system which is necessary for the secretion of harmful endogenous substances including antibiotics from the inner cytoplasm of the bacterial cell (Yang et al., 2021). The overexpression of efflux pumps reduces the effect of antibiotics resulting in antibiotic resistance in Gram-negative bacteria (Siroua *et al.* 2022). de Morais Oliveira-Tintino et al. (2018) performed a study on the inhibition of efflux activity from *Chenopodium ambrosioides* essential oil combined with ethidium bromide. The study concluded a lower MIC of ethidium bromide when used with the essential oil even though *Chenopodium ambrosioides* reported no antibacterial activity on its own. Therefore, suggesting essential oils may be a positive EPI (Efflux pump inhibitors) in the fight against antibiotic resistance.

2.6 DISK DIFFUSION

Disk diffusion was developed in the 1940's is an efficient method to screen the antimicrobial activity that of products such as essential oils or natural products (Nyiew, Kwong and Yow 2022). The surface of an agar plate is inoculated with seed inoculum and spread over the entire surface, a paper disk (disc) is placed onto the agar with a specific concentration of the antimicrobial solution to be tested (Hossain *et al.* 2022). The petri dish will then be incubated to allow the antibacterial properties to inhibit the bacterial growth. This inhibition of growth known as the zone of inhibition can then be measured to assess if the solution is antibacterial.

2.7 BROTH DILUTION

Broth macro-dilution or micro-dilution is used to identify the highest bactericidal and bacteriostatic activity (Nyiew, Kwong and Yow 2022). Bacteriostatic is the lowest concentration that is needed to inhibit the growth of bacteria also known as Minimal Inhibitory Concentration (MIC), whereas bactericidal is the lowest concentration that is needed to destroy the bacteria also known as Minimum Bactericidal Concentration (MBC) (Hossain *et al.* 2022).

2.8 RESPONSE SURFACE METHODOLOGY (RSM)

Response Surface Methodology (RSM) is a statistical design for analysing and optimizing dependant variables of an experimental process (Manojkumar, Muthukumaran and Sharmila 2022). This design uses a mathematical algorithm to effectively maximise the interactions of previously obtained data of variables, thereby allowing the response of results to be predicted for future study (Liu *et al.* 2022; Sirichan *et al.* 2022).

CHAPTER 3: METHODOLOGY

3.1 INTRODUCTION

This study adopted a quantitative approach guided by a true-experimental post-test only design. The experimental design was used to collect data in a controlled laboratory for the purpose of testing the hypothesis (DeCarlo 2018). The classification of a true experiment requires three specific criteria which applies to this study. The three criteria include a randomly assigned controlled group, a randomly assigned experiment group and the change of one variable (Williams 2020). In accordance with these prerequisites of quantitative research, this study included known number of identified bacteria in both the control and experiment group. Selected independent variables were changed in different experiments to analyse the effect on the dependant variable. Thereby testing the essential oil-based hand sanitizer against the chosen bacteria.

3.2 MATERIALS AND METHODS

3.2.1 ESSENTIAL OILS & BACTERIA

Pure essential oils of clove bud (C), lemongrass (LG) tea tree (TT), lavender, lemon, eucalyptus and peppermint were locally bought from Escentia Products (PTY) LTD - 2019/445189/07. Certificate of analysis can be found in Annexure 1, 2 & 3. The glass amber bottles of essential oils were stored in a dark box, in a secured storage cabinet.

All bacterial isolates used as test organisms (*Micrococcus luteus*, *Escherichia coli* ATCC 11775, *Klebsiella pneumoniae*, *Bacillus cereus* ATCC 10876 and *Bacillus subtilis*) were obtained from the culture collection of the Department of Biotechnology and Food Science, Durban University of Technology, Durban, South Africa. Both Gram-negative and Gram-positive bacteria were used for this investigation. The obtained bacteria were preserved onto nutrient agar (NA) slants at 4 ºC and in glycerol stocks at -80 ºC.

3.2.2 MEDIUM PREPARATION

The liquid medium, nutrient broth (NB, Biolab) was prepared by dissolving 16 g of nutrient broth powder in 1 L distilled water. Similarly, the solid medium, nutrient agar (NA) was prepared by dissolving 31 g of NA in 1 L of distilled water. The solution was sterilized in an autoclave at 121ºC for 15 minutes at 15 psi (pounds per square inch) pressure. Solid NA plates were prepared by cooling the sterilized NA medium to about 50 ºC and thereafter pouring aseptically into sterile Petri plates (approximately 25ml) and allowed to solidify at room temperature (25ºC).

3.2.3 PREPARATION OF BLANK DISCS

Whatman No. 3 filter paper was used to prepare blank discs. A disinfected office punch was used to make discs approximately 6 mm in diameter. The disks were placed into a glass beaker and autoclaved for at 121 ºC for 15 min at 15 psi.

3.2.4 PREPARATION OF ESSENTIAL OIL AND STOCK SOLUTION

Each EO was diluted to 10% (v/v) in NB containing 0.8% Tween 80. The solution was sonicated at 5 s on and 5 s off at 90% amplitude in an ultrasonicator (Sonics, USA) for 2 minutes for maximum emulsification. For consistency, the stock nutrient broth containing 0.8% (v/v) Tween 80 was also sonicated under similar conditions for 2 minutes.

3.3 GAS CHROMATOGRAPHY-MASS SPECTROSCOPY (GC-MS)

The composition of CEO, LGEO and TTEO were analysed using GC-MS by the Central Analytical Facilities (CAF) of Stellenbosch University, South Africa. Separation was performed on a gas chromatograph (6890N, Agilent technologies network) coupled to an Agilent technologies inert XL EI/CI Mass Selective Detector (MSD) (5975B, Agilent technologies Inc., Palo Alto, CA). The GC-MS system was coupled to a CTC Analytics PAL autosampler. Separation of the essential oils was performed a non-polar ZB-5Ms (30 m, 0.25 mm ID, 0.25 µm film thickness) capillary column.

The mass spectrometer was operated under electron impact (EI) mode at ionization energy of 70eV, scanning from 35 to 500m/z.Helium at a flow rate of 1 ml/min used as carrier gas.

3.4 ANTIBACTERIAL ACTIVITY: DISC DIFFUSION

Antibacterial activity of seven essential oils (Lavender, tea tree, lemon, lemon grass, clove, eucalyptus and peppermint) were tested using the disk diffusion method. Briefly, the bacterial cultures were revived by transferring a loop of cells from the NA slant and quadrant streaked onto a sterile NA petri plate where it was incubated at 37 ºC for 16 h. After incubation at 37 ºC for 16h, single colony of separate bacteria were transferred aseptically from the NA plate into different test tubes containing 20 ml of NB. The test tubes were incubated in a shaking incubator at 37 ºC for 16 h at 150 rpm (revolutions per minute). Sterile NA plates were divided into 6 equal sections and labelled according to the essential oil to be tested for the antibacterial activity. Thereafter, 100 µl of the seed culture inoculum was spread across the plate using a sterilized spreader. Sterile Whatman paper 3 disks were saturated with 10 µl of 100% of the respective EOs. All plates contained a 70% ethanol disc as the positive control and distilled water (sterilized) disc as the negative control. The plates were then placed in an incubator at 37 ºC for 24 h. All experiments were conducted in triplicate a level 2 biosafety laminar flow.

3.5 MINIMUM BACTERICIDAL CONCENTRATIONS

The bacterial culture was revived by transferring a loop of cells from the NA slant and quadrant streaked onto a sterile NA Petri plate where it was incubated at 37 ºC for 16 h. Thereafter, single colony of cells were aseptically transferred from the NA plate into test tube containing 20 ml of NB where it was incubated in a shaking incubator at 37 ºC for 16 h, 150 rpm.

One ml of serial dilutions (v/v) of selected EOs (10%, 5%, 2.5%, 1%, 0.75%, 0.5%, 0.25%, 0.125%) were made in 2 ml microcentrifuge tubes. Each tube was inoculated with 100 µl of seed inoculum of respective bacteria (except for the negative control) and incubated at 37 ºC for 16 h at 150 rpm. After 16 h, 100 µl was spread plated onto sterile petri plates in triplicates containing

NA and incubated for 24 h at 37 ºC for observation of the concentration when no visible growth was seen.

3.6 RESPONSE SURFACE METHODOLOGY USING CENTRAL COMPOSITE DESIGN (CCD)

Three essential oils, LGEO, TTEO and CEO, which significantly influenced the growth of bacteria were selected as independent variables. The optimum levels of the three selected variables and their interactions were studied as per the experimental design constructed by Design Expert 6.0 (Stat-Ease Inc., Minneapolis, USA). Each variable was studied at five different levels ($-\alpha$, -1, 0, +1, $+\alpha$) to determine their optimal values and examine their interactions as per CCD, with a total of 20 experimental runs.The behaviour of the system was explained by the following second order polynomial equation:

$$\mathbf{Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD} \quad \text{Equation 1}$$

where Y is log reduction of bacteria as the predicted value of response. The log reduction Y was defined as $\log N_0/N$, where $\log N_0$ was initial CFU/ml before treatment and N was final CFU/ml after using EOs in the blended hand sanitizer formulation. β_0 is intercept, β_1, β_2, β_3, β_4 are linear coefficients, β_{11}, β_{22}, β_{33}, β_{44} are squared quadretic coefficients, β_{12}, β_{13}, β_{23}, β_{24}, β_{34} are interaction coefficients and A, B, C, A^2, B^2, C^2, D^2, AB, AC, BC, BD,CD are independent variables.

CHAPTER 4: RESULTS

4.1 CHEMICAL COMPOSITION OF ESSENTIAL OILS: GC-MS ANALYSIS

Multiple volatile chemical compounds were observed after GC-MS analysis of LGEO, CEO and TTEO. As expected, different EOs showed entirely different groups of chemical compounds. Fig. 4.1 shows the GC-MS analysis of LGEO, which identified a total of 48 chemical compounds. (table A1). The percentage of each compound can be seen in Fig 4.2. Major compounds identified in LGEO were geranial (43.88%), neral (28.38%), neryl acetate (5.39%) and β-caryophyllene (2.84%) [table 4.1]. Analysis of CEO as seen in Fig. 4.3 presented with 17 chemical compounds (Table A2) with the major being eugenol (73.50%), eugenol acetate (17.38%) and β-caryophyllene (5.72%) as shown in table 4.2. The total percentage of each compound identified can be seen in Fig 4.4. TTEO analysis as seen in Fig 4.5 presented with 52 chemical compounds (Table A3) with the major chemical compounds being terpineol-4, γ-terpinene, α-terpipene and 1,8-cineole (eucalyptol) as seen in table 4.3. The total percentage of each compound identified can be seen in Fig 4.6.

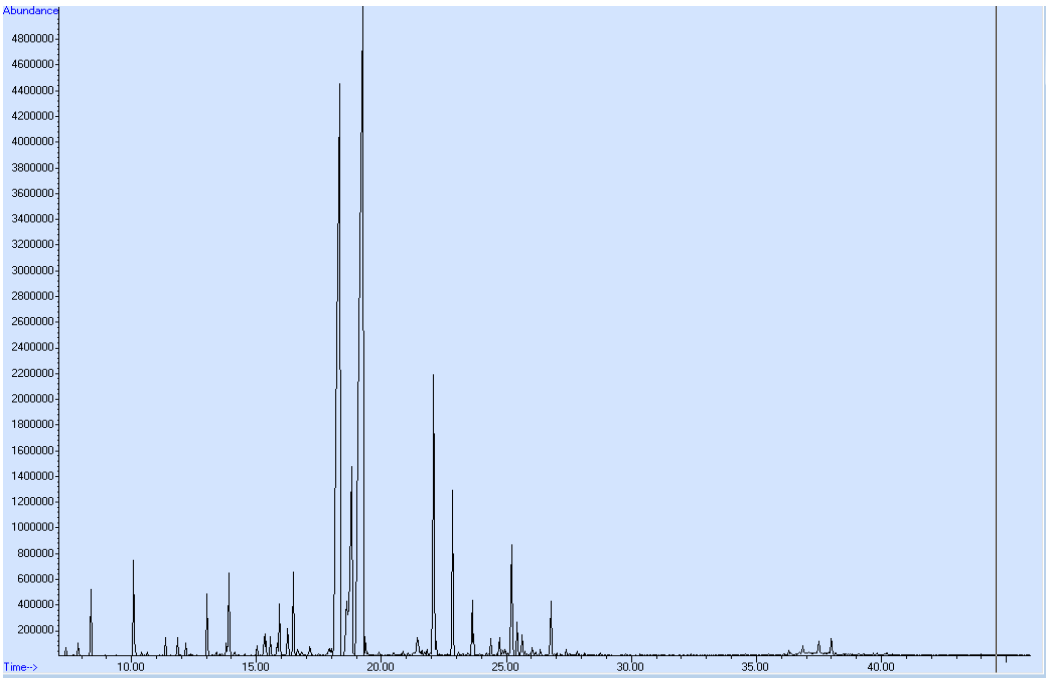


Fig. 4.1 GC-MS chromatogram of LGEO using a non-polar ZB-5Ms capillary column.

Table 4.1 GC-MS analysis showing percentage concentration of VOCs in lemongrass

Retention Time (min)	Compound Identified	Percent (%)
19.26	geranial	43.88
18.34	neral	28.38
22.10	neryl acetate	5.39
22.85	β-caryophyllene	2.84

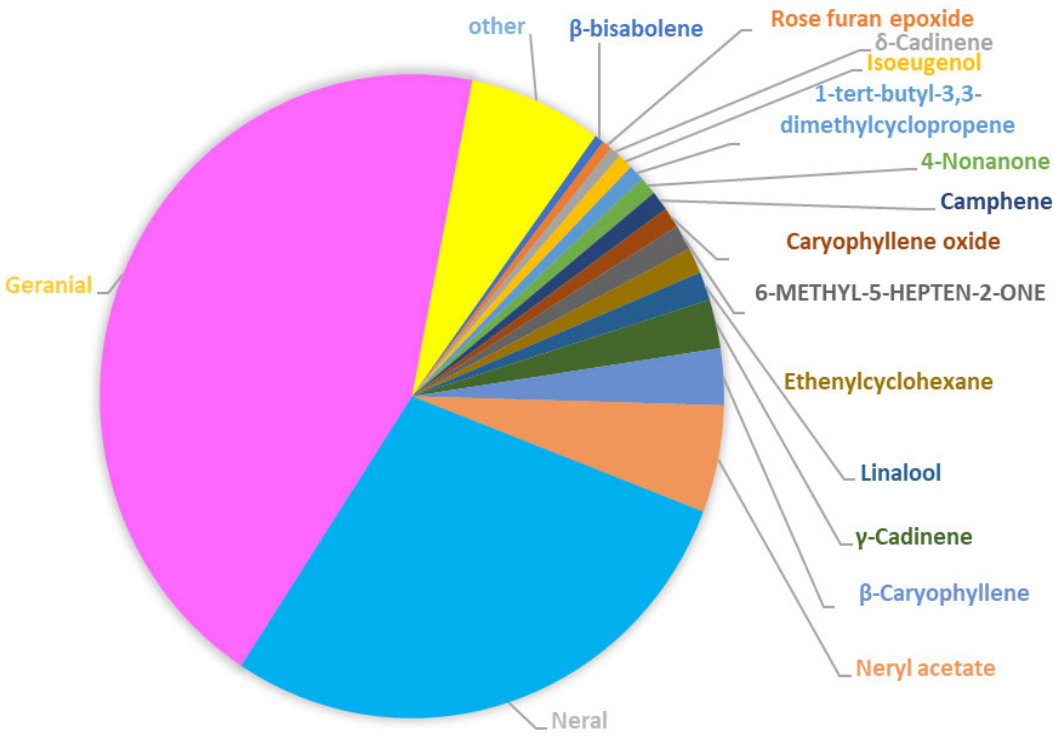


Fig 4.2 Chemical compound percentage analysis of LGEO

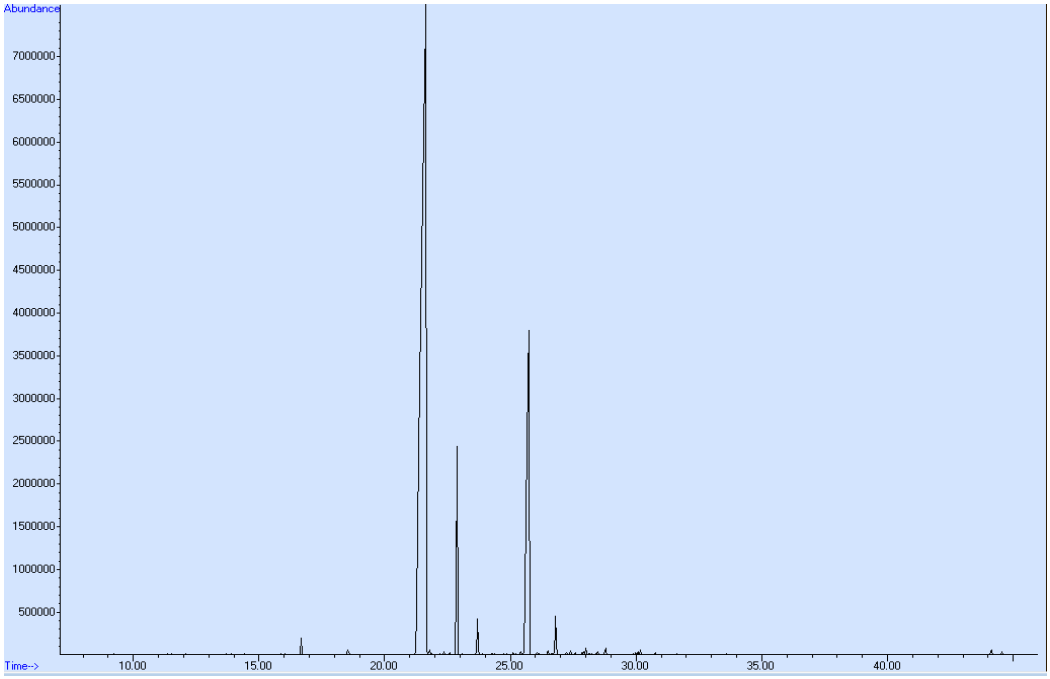


Fig. 4.3 GC-MS chromatogram of CEO using a non-polar ZB-5Ms capillary column.

Table 4.2 GC-MS analysis showing percentage concentration of VOCs in CEO

Retention time (min)	Compound identified	Percent (%)
21.65	eugenol	73.50
25.73	eugenyl acetate	17.38
22.89	β-caryophyllene	5.72

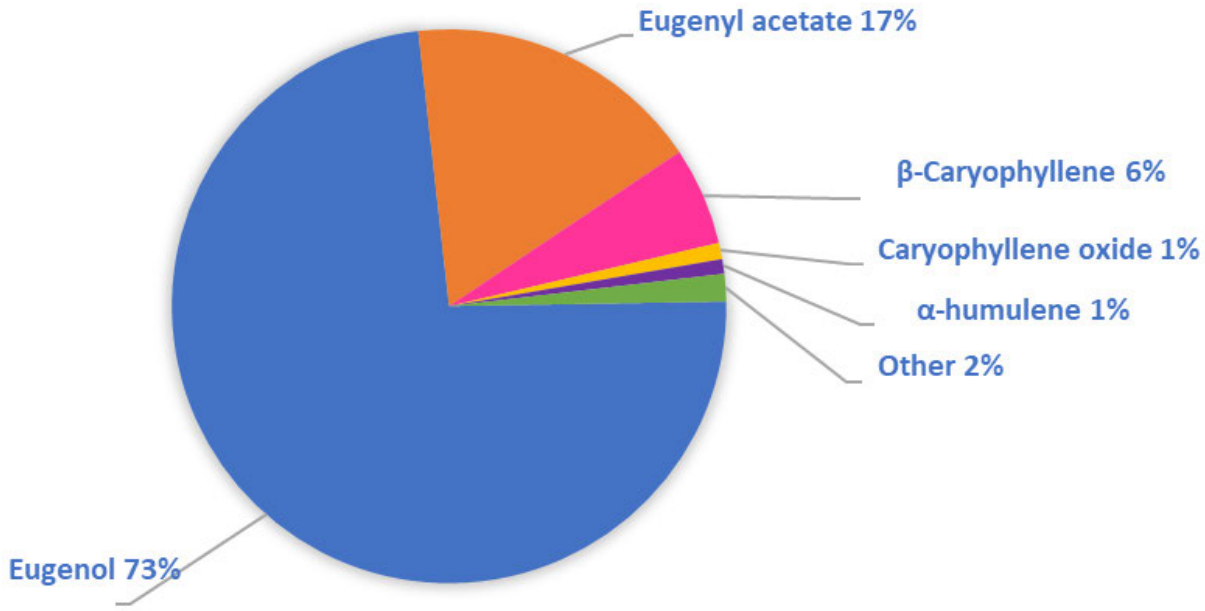


Fig 4.4 Chemical compound percentage analysis of clove essential oil

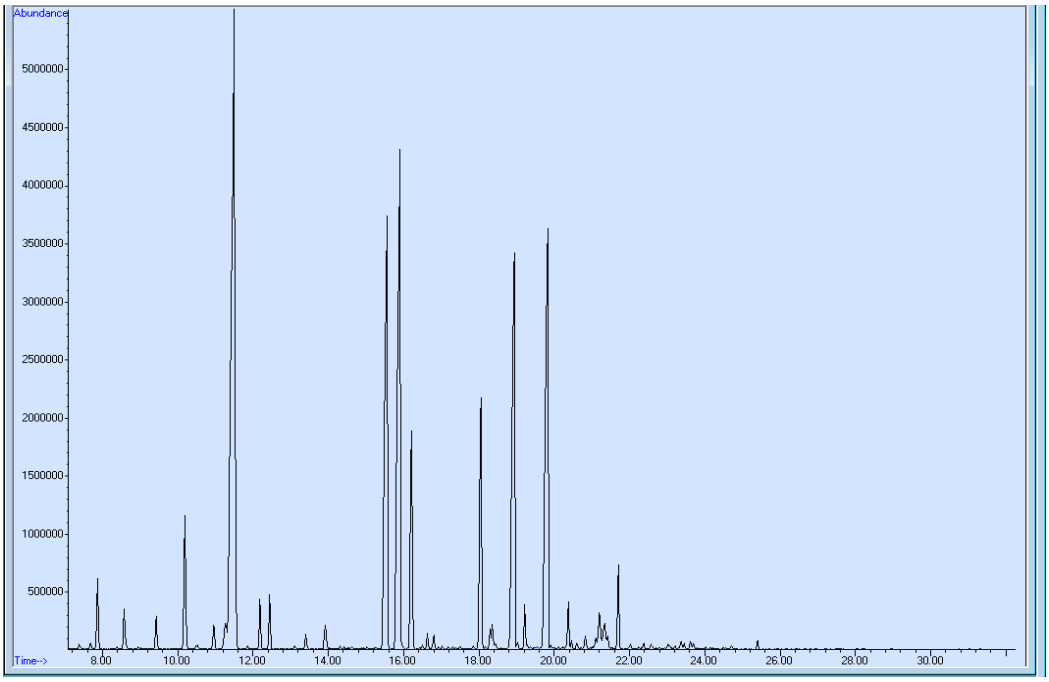


Fig. 4.5 GC-MS chromatogram of tea tree oil using a non-polar ZB-5Ms capillary column.

Table 4.3 GC-MS analysis showing percentage concentration of VOCs in tea tree oil

Retention time (min)	Compound identified	Percent (%)
16,4741	terpineol-4	36.39
12,589	γ -terpinene	19.12
11,0421	α-terpipene	10.83
11,4764	1,8-cineole (eucalyptol)	3.22

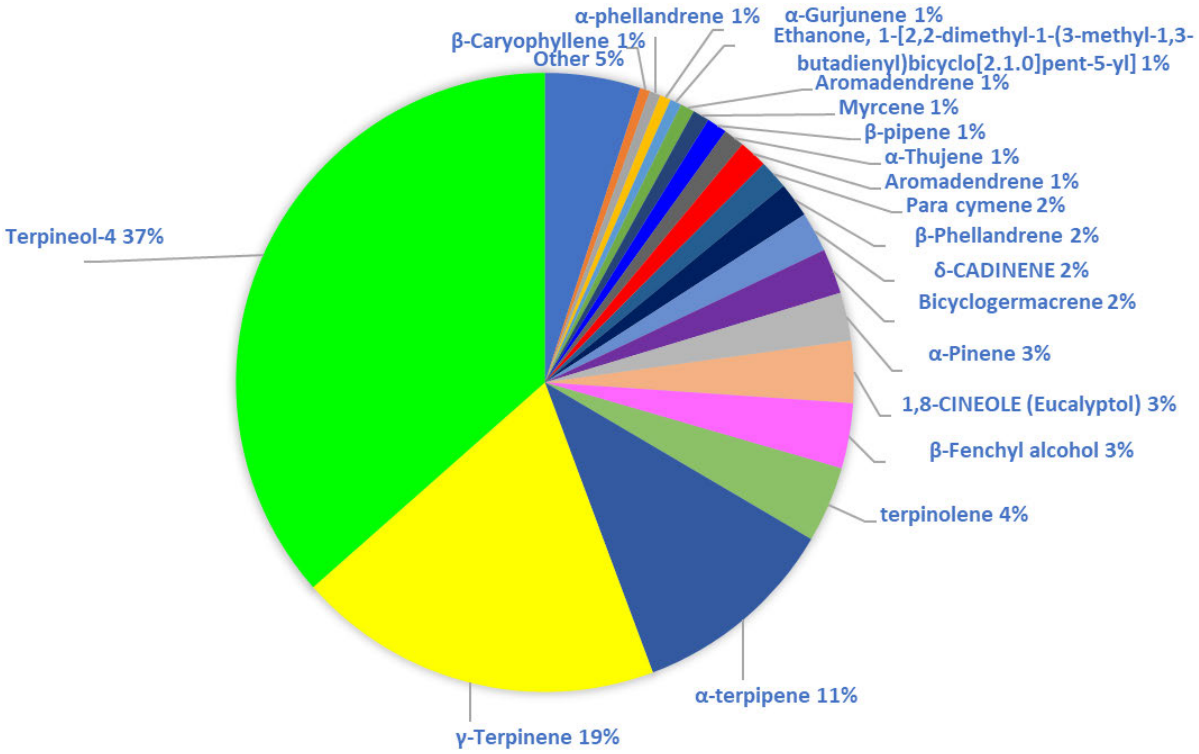


Fig 4.6 Chemical compound percentage analysis of TTEO

4.2 ASSESSMENT OF ANTIBACTERIAL ACTIVITY OF DIFFERENT EOs

The antibacterial activity of different EOs was tested against different bacteria to select the most effective EOs for further studies.

Fig. 4.7 Antibacterial activities of clove (C), eucalyptus (E), peppermint (P) and tea tree (T) EOs against *M. luteus.*

Fig. 4.8 Antibacterial activities of lavender (L1), lemongrass (L2) and lemon (L3) essential oil against *M. luteus.*

Fig. 4.7 and 4.8 demonstrate the ZOI of EOs against *M. luteus*. In Fig 4.7, C1 and C2 were controls with no ZOI, eucalyptus EO was designated as E, which was not effective against *M. luteus.* P represented peppermint EO and T represented tea tree EO which showed a small zone of inhibition. Clove essential oil was represented by C which showed a large zone of inhibition against *M. luteus.* Fig. 4.8 illustrates that *M. luteus* was not sensitive against lavender EO. However, it showed slight sensitivity with a small ZOI against lemon EO while a large ZOI was observed against LGEO indicating highest sensitivity.

Fig. 4.9 Antibacterial activities of lavender (L1), lemongrass (L2) and lemon (L3) EOs against *B. subtilis*

Fig. 4.10 Antibacterial activities of clove (C), eucalyptus (E), peppermint (P) and tea tree (T) EOs against *B. subtilis*

Fig. 4.9 shows that lemon (L3) and lavender (L1) EO had little effect on *B. subtilis* with very small ZOI. LG (L2) oil showed a very large ZOI against *B. subtilis*. In comparison, Fig. 4.10 shows no activity of eucalyptus (E) oil against *B. subtilis* whereas peppermint (P) and tea tree (T) showed a slight effect on *B. subtilis.* Although clove (C) essential oil emerged as a potential antibacterial EO but its activity was not as effective as LGEO.

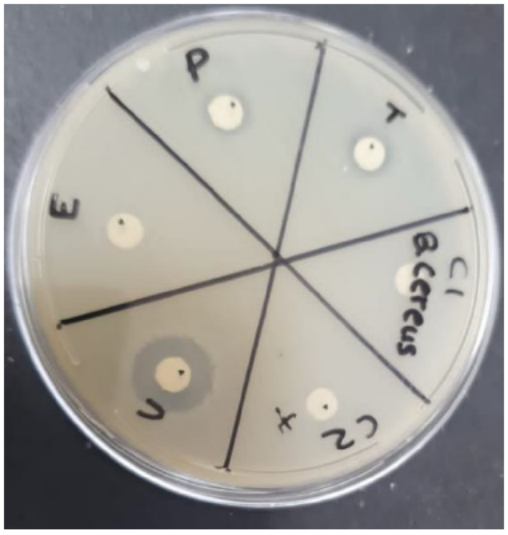


Fig. 4.11 Antibacterial activities of clove (C), eucalyptus (E), peppermint (P) and tea tree (T) EOs against *B. cereus*

Fig. 4.12 Antibacterial activities of lavender (L1), lemongrass (L2) and lemon (L3) EOs against *B. cereus*

Eucalyptus (E) displayed no antibacterial activity against *B. cereus* as seen in Fig. 4.11. Peppermint (P) and tea tree (T) essential oils showed slight sensitivity against *B. cereus*. CEO showed a clear ZOI against *B. cereus*. Lemon (L3) and lavender (L1) showed slight antibacterial effects against *B. cereus* with a small ZOI as seen in Fig. 4.12. LG (L2) had the greatest antibacterial effect against *B. cereus* with a large zone of clearance.

Fig. 4.13 Antibacterial activities of lavender (L1), lemongrass (L2) and lemon (L3) EOs against *K. pneumoniae*

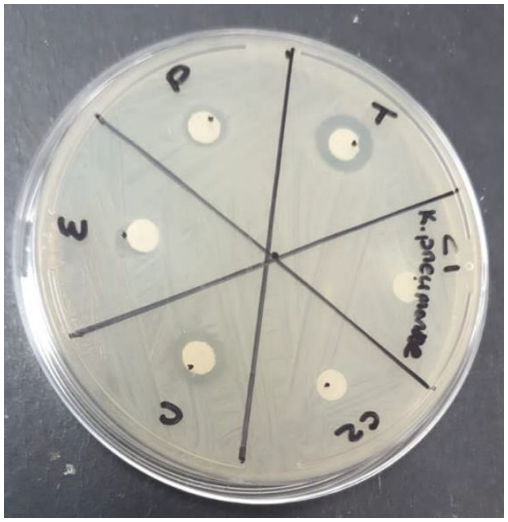


Fig. 4.14 Antibacterial activities of clove (C), eucalyptus (E), peppermint (P) and tea tree (T) EOs against *K. pneumoniae*

The antibacterial activity of lemon (L3) EO against *K. pneumoniae* showed no ZOI as seen in Fig. 4.13 lavender (L1) and LG (L2) have display a small zone of clearance. Eucalyptus (E) as seen in Fig. 4.14 showed no antibacterial sensitivity against *K. pneumoniae*. Peppermint (P) EO had a very small zone of clearance against *K. pneumoniae* with CEO displaying a slightly larger ZOI against *K. pneumonia*e. However, tea tree (T) EO showed the largest, distinct ZOI against *K. pneumoniae*.

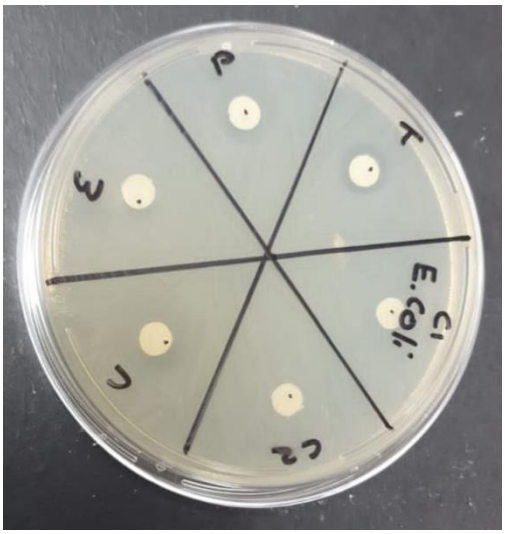


Fig. 4.15 Antibacterial activities of clove (C), eucalyptus (E), peppermint (P) and tea tree (T) EOs against *E. coli*

Fig. 4.16 Antibacterial activities of lavender (L1), lemongrass (L2) and lemon (L3) EOs against *E. coli*

CEO as seen in Fig. 4.15 demonstrates a ZOI against *E. coli* ATCC 11775. Eucalyptus essential oil had no effect whereas peppermint and tea tree EO has a small effect with a small ZOI. Lavender (L1) as seen in Fig. 4.16 had no impact on *E. coli* ATCC 11775, compared to LG (L2) which had a large zone of clearance. lemon (L3) which had a slight zone of clearance. Table 4.4 demonstrates the ZOI is depicted throughout figures 4.7 through to 4.16:

Table 4.4 Zone of inhibition due to different essential oils

Name of bacteria	**Zone of inhibition (mm)**						
	LGEO	**CEO**	**TTEO**	**Lemon EO**	**Lavender EO**	**Peppermint EO**	**Eucalyptus EO**
M. luteus	13.5	20.4	7.4	7.1	0.0	7.3	0.0
B. subtilis	30.2	13.4	7.8	7.5	6.8	7.7	0.0
B. cereus	41.4	15.0	8.0	7.8	8.1	8.4	0.0
K. pneumoniae	9.0	10.7	11.5	7.6	7.1	8.6	7.6
E. coli	18.9	12.1	9.1	8.1	0.0	11.4	0.0

4.3 MINIMUM BACTERICIDAL CONCENTRATION (MBC) OF ESSENTIAL OILS

Essential oils were individually tested against certain bacteria to determine their MBCs. LGEO proved to be the most effective against the selected bacteria as seen in table 4.5 with the most effective against *Micrococcus luteus* with an MBC of less than 0.125%.

Table 4.5 Minimum Bactericidal Concentration (MBC) of Essential Oils

Bacteria	**Lemongrass Essential Oil**	**Tea tree Essential Oil**	**Clove Essential Oil**
K. pneumoniae	0.5%	1%	0.5%
M. luteus	< 0.125%	0.75%	0.5%
E. coli ATCC 11775	0.5%	0.75%	0.5%
B. cereus	10%	>10%	>10%
B. subtilis	2.5%	>10%	>10%

4.4 STATISTICAL OPTIMIZATION USING RSM

RSM using CCD was applied to determine the optimal levels of the selected essential oils as independent variables (LGEO, TTEO and CEO), which significantly influenced the growth of bacteria (dependent variable). The optimum levels of the three selected variables and their interactions were studied as per the experimental design shown in table 4.6. (Table 4.6 *a*) shows the experimental range and level of independent variables which shows the lowest (– 2), central (0) and highest (– 2) levels of variables to be used in CCD. A total of 20 experiments were carried out using several combinations of the variables as per the CCD. The observed responses of the CCD experiments are presented in (Table 46 *c*), with the highest being in run 8 and the lowest being in run 1. Results of the experiments were analysed by standard ANOVA (Table 2.2c). The following second-order polynomial equation was obtained that explains phytase production as a function of the four variables:

$$\mathbf{Y = +5.57 + 1.11\,A + 0.63\,B + 0.51\,C - 0.51A^2 - 0.44\,B^2 - 0.32\,C^2 + 0.015AB - 0.16\,AC - 0.078\,BC}$$

Equation 2

Table 4.6 Experimental range (a) experimental design (b) and ANOVA analysis (c) for optimization of EOs for enhanced log reduction using response surface methodology

(a) Experimental range and level of independent variables

Variables	**Symbol**	**Range and level**				
		-α	**-1**	**0**	**+1**	**+α**
LGEO (%)	x_1	0.06	0.07	0.09	0.11	0.12
TTEO (%)	x_2	0.06	0.07	0.09	0.11	0.12
CEO (%)	x_3	0.06	0.07	0.09	0.11	0.12

(b)Experimental design and response for RSM studies

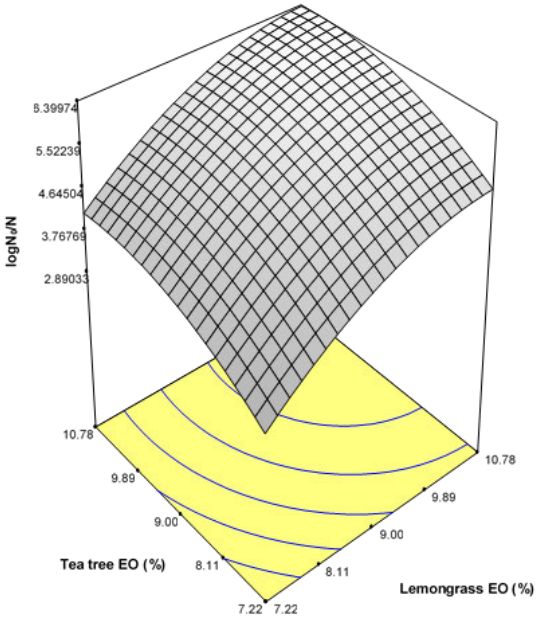
Run no.	**Values of variables**			**$LogN_0/N$**
	x_1	**x_2**	**x_3**	
1	7.2	7.2	7.2	1.77
2	10.8	7.2	7.2	4.29
3	7.2	10.8	7.2	3.33
4	10.8	10.8	7.2	5.76
5	7.2	7.2	10.8	3.28
6	10.8	7.2	10.8	5
7	7.2	10.8	10.8	4.38
8	10.8	10.8	10.8	6.31
9	6.0	9.0	9.0	2.22
10	12.0	9.0	9.0	6.1
11	9.0	6.0	9.0	3.42
12	9.0	12.0	9.0	5.32
13	9.0	9.0	6.0	3.78
14	9.0	9.0	12.0	5.64
15	9.0	9.0	9.0	5.57
16	9.0	9.0	9.0	5.55
17	9.0	9.0	9.0	5.56
18	9.0	9.0	9.0	5.57
19	9.0	9.0	9.0	5.57
20	9.0	9.0	9.0	5.57

(c) ANOVA values

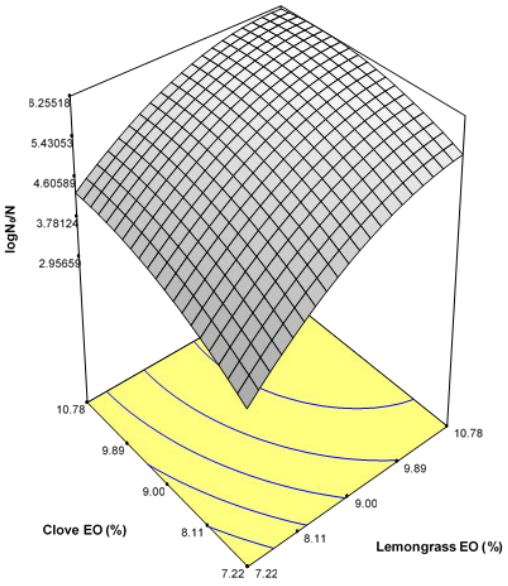
Source	**Sum of Squares**	**Degree of freedom**	**Mean Square**	***F*-value**	**P-value (Prob>F)**	**Significance**
Model	32.79	9	3.64	318.21	< 0.0001	significant
Residual	0.11	10	0.011			
Total	32.91	19				

[†]R^2 = 0.9965; Adjusted R^2 = 0.9969; Pred R^2 = 0.9934

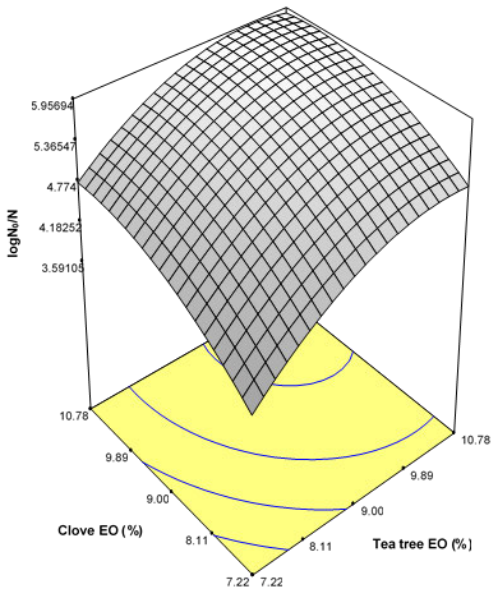
The model F-values of 318.21 and 'Prob>F value' of < 0.0001 implied the model was highly significant. The quadratic effects of LGEO (A^2), TTEO (B^2) and CEO (C^2), as well as the interaction between LGEO and CEO (AC) and TTEO and CEO (BC) revealed a significant effect on the overall log reduction of the bacteria.



(a)



(b)



(c)

Fig 4.17 Response surface contour of the interaction between tea tree EO and lemongrass EO (a), clove EO and lemongrass EO (b), and clove EO and tea tree EO (c).

CHAPTER 5:

5.1 GC-MS ANALYSIS OF ESSENTIAL OILS

GC-MS is a powerful analytical technique for the identification of volatile molecules. This preferred method is able to identify multiple components in a compound (Sobhy *et al.* 2023).

GC-MS analysis identified a total of 48 compounds in LGEO. The results are in accordance with Mahmoud *et al.* (2022) where the authors also identified geranial, neral, neryl acetate and β-caryophyllene (2.84%) as the major constituents. However, Mahmoud *et al.* (2022) reported only 31 compounds. (Mahmoud *et al.* 2022). The total of geranial and neral equals 72.26%, which is primarily composed of monoterpene aldehydes (Kumoro *et al.* 2021). More recently, Recio-Cázares *et al.* (2023) reported high levels of monoterpene in *Aloysia citriodora* essential oil and attributed to antimicrobial effects.

GC-MS analysis of CEO identified a total of 17 volatile compounds. The major compounds being eugenol (73.5%), eugenol acetate (17.38%) and β-caryphyllene (5.72%). Interestingly, the same major compounds were identified by Chen *et al.* (2022). However, this study reports higher concentrations of eugenol and eugenol acetate (73.5% and 17.38%) than Chen *et al.* (2022) (43.5% and 8.73%, respectively). Conversely, β-caryphyllene was only 5.72% as compared to 36.34% reported by Chen *et al.* (2022). It is evident that the dominating chemical compound in CEO is eugenol, which is known to exhibit marked antibacterial properties (Di Consiglio *et al.* 2023). Eugenol is known to disrupt the cell membrane resulting in the swelling of cells. Furthermore, membrane hyperpolarization due to eugenol enhances the membrane permeability (Ulanowska and Olas, 2021).

GC-MS was used to identify 52 compounds of TTEO. Terpinene-4-ol was identified as the major compound in this study, which was in accordance with the previous study by Chintalchere *et al.* (2021). However, the top 5 major compounds identified in this study differ from the 5 major compounds identified by Chintalchere *et al.* (2021). This study identifies terpinene-4-ol (36.39%), γ-terpinene (19.12%), α-terpipene (10.83%) and 1,8-cineole (also known as eucalyptol) (3.22%) as the major compounds while Chintalchere *et al.* (2021) reported terpinen-4-ol (45.78%), γ-terpinene (16.52%), 4-carene (8.75%) and β-cymene (7.98%) and a small proportion of α-terpineol

(4.48%) in TTEO. Terpineol-4-ol and its derivatives are known for its antibacterial effects (Sobhy *et al.* 2023).

Majority of the essential oils that have been analysed via GC-MS when compared to the same type of essential oil have the same major volatile compounds however, the quantities differ vastly between the oils. This will influence the desired outcome that is expected of the essential oil. This difference may be due to the area the plant has been harvested, the time from the time of harvest to the time of processing, the method of extraction that was used to extract the essential oil, the storage of the essential oil, or the time taken from the processed product being used.

5.2 MIC AND MBC OF ESSENTIAL OILS

The MIC was used *in* vitro to identify the antibiotic effects of essential oils against bacterial strains. This study identified LEO effective against *K. pneumoniae, Micrococcus luteus and E. coli* with an MIC of 0.5%, 0.125% and 0.5% (V/V) respectively. Luis A. Ortega-Ramirez et al. (2020) demonstrated lower mechanism of inhibition of LGEO against *E. coli*. Citral, and geraniol present in LGEO showed MIC of 2.2 and 1.0mg/mL, respectively against *E. coli*. In contrast to the present study, tea tree and thyme essential oils were more efficient than lemongrass as reported by Loose, Pilger and Wagenlehner (2020). Torres Neto *et al.* (2022) used a combination of oregano (50%), thyme (40%), and lemongrass (10%) EOs to achieve maximum inactivation of foodborne pathogens. In a study by Pedrós-Garrido *et al.* (2020) LGEO was effective against *L. monocytogenes* showing MIC of 0.4% (V/V). In this study CEO demonstrated a consistent MIC of 0.5% (V/V) against *K. pneumoniae, Micrococcus luteus and E. coli.* This is supported by a study by Pathirana *et al.* (2019) whereby MBC for Gram-negative bacteria ranged from 0.25-0.5% (V/V). The same study reported MBC for Gram-positive bacteria ranging between 0.25-1% (V/V). Even though CEO is effective against *E. coli* Xiao *et al.* (2019) reported that oregano was more effective against E. coli with an MIC of 0.035% compare to clove that had an MIC of 0.125%. Griffin, Markham and Leach (2000) studied the MIC of TT EO against *S. aureus*, *S. epidermidis*, *B. subtilis*, *B. cereus*, *Micrococcus luteus*, *Streptococcus*, *E. coli*, *Pseudomonas*, and *Proteus* ranged between 0.2% and 0.5% (V/V). This was slightly more effective than this

study which had an MIC of 0.75% (V/V) TT EO against *E. coli* as well as *Micrococcus luteus.* In a different study, Borotová *et al.* (2022) identified the MIC 90 (µl/ml) for B. subtilis 18.36, Micrococcus luteus 18.68; for Gram-negative ranged from 12.32 to 25.42.

5.3 STATISTICAL OPTIMIZATION USING RSM

RSM using central composite design (CCD) was employed for statistical optimization of the selected essential oils and to study their interactions for enhanced log reduction of bacteria. Concentrations of LGEO, TTEO and CEO were used as three independent variables and their interaction were studied at five different levels (-α was lowest level, -1, 0 was central level, +1, +α was highest level). Response in the form of bacterial log reduction ranged from 3.33 ± 0.08 to 6.31 ± 0.19. Experimental run 11 yielded maximum log reduction of 6.31 and experiment run one yielded minimum log reduction of 3.33. The synergistic effect of LGEO, TTEO and CEO resulted in enhanced inhibition of *E. coli* growth. The optimized blend of LGEO, TTEO and CEO contained 0.10%, 0.11% and 0.10%, respectively. Similar effects on log reduction of all Gram-positive and Gram-negative bacteria were observed when statistically optimized concentrations of EO blend was used during validation experiments (data not shown).

Cattelan et al. (2018) developed RSM model to study the interaction of oregano EO and salt concentrations on the growth of *E. coli* in salad dressing. Sani et al. (2019) used RSM to show the enhanced antibacterial properties of composite film against *E. coli* by increasing the concentration of Melissa EO and zinc oxide. Similarly, Azevedo et al. (2014) used RSM to develop a mathematical model to achieve enhanced zone of inhibition against *S. aureus, B. subtilis, S. marcescens* and *S. enteritidis* as a function of concentrations of starch, chitosan and EO. Although there is sufficient literature available on the use of RSM for enhanced production of essential oils, reports on the use of RSM for log reduction of bacteria is limited. In fact, to the best of our knowledge, this is the first report on the use of RSM for statistical optimization of LGEO, TTEO and CEO for enhanced log reduction of bacteria.

5.3 CONCLUSION

The complex compounds of LG, TT and C EOs were tested for their Minimum inhibitory concentrations against common Gram-positive and Gram-negative bacteria. These oils were proven to have powerful antibacterial properties in low concentrations. The optimized essential oils had a synergistic effect once combined, which further enhanced their antibacterial properties at even lower concentrations. We can therefore conclude that the low concentrations of essential oils may assist in preventing the spread of bacteria causing disease.

5.4 FUTURE PERSPECTIVES

This study noted potential for further investigation whereby essential oils may be tested against a wider range of pathogenic bacteria. The efficacy of essential oils against viruses, fungi and nematodes will also provide useful scientific knowledge. Furthermore, studies aimed at animal models and biomimetics should be performed to test the safety of essential oils on humans. Lastly, there should be more studies on the different combinations of activate chemical components in essential oils to investigate their effect on pathogenic cell disruption.

REFERENCES

Aćimović, M. 2021. Essential oils: Inhalation aromatherapy—a comprehensive review. *Journal of Agronomy, Technology and Engineering Management*, 4: 547-557. Available: https://www.researchgate.net/profile/Milica-Acimovic/publication/349485550_Essential_Oils_Inhalation_Aromatherapy_-A_Comprehensive_Review/links/6032d264a6fdcc37a84249c8/Essential-Oils-Inhalation-Aromatherapy-A-Comprehensive-Review.pdf (Accessed 27 July 2022).

Aimad, A., Sanae, R., Anas, F., Abdelfattah, E. M., Bourhia, M., Mohammad Salamatullah, A., Alzahrani, A., Alyahya, H. K., Albadr, N. A. and Abdelkrim, A. 2021. Chemical Characterization and Antioxidant, Antimicrobial, and Insecticidal Properties of Essential Oil from Mentha pulegium L. *Evidence-Based Complementary and Alternative Medicine*, 2021. Available: https://www.hindawi.com/journals/ecam/2021/1108133/ (Accessed 28 July 2022).

Ak Sakallı, E., Teralı, K., Karadağ, A. E., Biltekin, S. N., Koşar, M., Demirci, B., Hüsnü Can Başer, K. and Demirci, F. 2022. In vitro and in silico evaluation of ACE2 and LOX inhibitory activity of Eucalyptus essential oils, 1, 8-cineole, and citronellal. *Natural Product Communications*, 17 (6): 1934578X221109409. Available: https://journals.sagepub.com/doi/pdf/10.1177/1934578X221109409 (Accessed 14 February 2023).

Aljaafari, M. N., AlAli, A. O., Baqais, L., Alqubaisy, M., AlAli, M., Molouki, A., Ong-Abdullah, J., Abushelaibi, A., Lai, K.-S. and Lim, S.-H. E. 2021. An Overview of the Potential Therapeutic Applications of Essential Oils. *Molecules*, 26 (3): 628. Available: https://www.mdpi.com/1420-3049/26/3/628 (Accessed 27 July 2022).

Aljaafari, M. N., Alkhoori, M. A., Hag-Ali, M., Cheng, W.-H., Lim, S.-H.-E., Loh, J.-Y. and Lai, K.-S. 2022. Contribution of aldehydes and their derivatives to antimicrobial and immunomodulatory activities. *Molecules*, 27 (11): 3589. Available: https://www.researchgate.net/profile/Richard-Herman-5/publication/336305801_Essential_Oils_and_their_applications-A_mini_review/links/5e92a663299bf1307991511f/Essential-Oils-and-their-applications-A-mini-review.pdf (Accessed 19 February 2023).

Angane, M., Swift, S., Huang, K., Butts, C. A. and Quek, S. Y. 2022. Essential Oils and Their Major Components: An Updated Review on Antimicrobial Activities, Mechanism of Action and Their Potential Application in the Food Industry. *Foods*, 11 (3): 464. Available: https://www.mdpi.com/2304-8158/11/3/464 (Accessed 25 July 2022).

Anisha Bhatia. 2021. *Hand Hygiene – Why, How & When. A Quick Guide*. Available: https://swachhindia.ndtv.com/swasthbharat-hand-hygiene-why-how-when-a-quick-guide-62872/ (Accessed 23 May 2022).

Aryal, S. 2022. *HPLC- Definition, Principle, Parts, Types, Uses*. Available: https://microbenotes.com/high-performance-liquid-chromatography-hplc/ (Accessed 25 March 2022).

Asteggiano, A., Occhipinti, A., Capuzzo, A., Mecarelli, E., Aigotti, R. and Medana, C. 2021. Quali–Quantitative Characterization of Volatile and Non-Volatile Compounds in Protium heptaphyllum (Aubl.) Marchand Resin by GC–MS Validated Method, GC–FID and HPLC–HRMS2. *Molecules*, 26 (5): 1447. Available: https://www.mdpi.com/1420-3049/26/5/1447 (Accessed 21 July 2022).

Banerjee, K., Madhyastha, H., Sandur V, R., N.T, M., N, T. and Thiagarajan, P. 2020. Anti-inflammatory and wound healing potential of a clove oil emulsion. *Colloids and Surfaces B: Biointerfaces*, 193: 111102. Available: https://doi.org/10.1016/j.colsurfb.2020.111102 (Accessed 15 May 2022).

Barnes, K. M., Whiffin, A. L. and Bulling, M. T. 2019. A preliminary study on the antibacterial activity and insect repellent properties of embalming fluids from the 18th Dynasty (1550–1292 BCE) in ancient Egypt. *Journal of Archaeological Science: Reports*, 25: 600-609. Available: https://www.sciencedirect.com/science/article/pii/S2352409X19300136 (Accessed 14 October 2021).

Barros, A. F., Campos, V. P., de Paula, L. L., de Oliveira, D. F. and Silva, G. H. 2020. Synergism of citral from Cymbopogon citratus essential oil and undecan-2-one against Meloidogyne incognita. *Nematology*, 22 (10): 1101-1110. Available: https://www.researchgate.net/publication/281903562_Anthelmintic_activity_of_Cymbopogon_citratus_against_Haemonchus_contortus (Accessed 20 February 2023).

Basyir, F., Munawar, A. and Aisyah, Y. 2021. Near infrared reflectance spectroscopy: classification and rapid prediction of patchouli oil content. In: Proceedings of *IOP Conference Series: Earth and Environmental Science*. IOP Publishing, 012071. Available: https://iopscience.iop.org/article/10.1088/1755-1315/667/1/012071 (Accessed 31 August 2022).

Booq, R. Y., Alshehri, A. A., Almughem, F. A., Zaidan, N. M., Aburayan, W. S., Bakr, A. A., Kabli, S. H., Alshaya, H. A., Alsuabeyl, M. S., Alyamani, E. J. and Tawfik, E. A. 2021. Formulation and Evaluation of Alcohol-Free Hand Sanitizer Gels to Prevent the Spread of Infections during Pandemics. *International Journal of Environmental Research and Public Health*, 18 (12): 6252. Available: https://www.mdpi.com/1660-4601/18/12/6252 (Accessed 18 August 2022).

Borotová, P., Galovičová, L., Vukovic, N. L., Vukic, M., Tvrdá, E. and Kačániová, M. 2022. Chemical and Biological Characterization of Melaleuca alternifolia Essential Oil. *Plants,* 11 (4): 558. Available: https://www.mdpi.com/2223-7747/11/4/558 (Accessed 19 July 2023).

Businesswire. 2019. *Global Tea Tree oil Market Analysis 2018-2025: Segmented by Application, End-user, Grade, and Region* Available: https://www.businesswire.com/news/home/20190828005321/en/Global-Tea-Tree-Oil-Market-Analysis-2018-2025-Segmented-by-Application-End-user-Grade-and-Region---ResearchAndMarkets.com (Accessed 28/01/2022).

Chakraborty, A., Mukhopadhyay, A., Sur, A. and Ghosh, S. 2021. Production of cheap hand sanitizer with herbal ingredients. *Journal of Physics: Conference Series,* 1797 (1): 012036. Available: https://iopscience.iop.org/article/10.1088/1742-6596/1797/1/012036/pdf (Accessed 18 August 2022).

Chen, Y., Xu, F., Pang, M., Jin, X., Lv, H., Li, Z. and Lee, M. 2022. Microwave-assisted hydrodistillation extraction based on microwave-assisted preparation of deep eutectic solvents coupled with GC-MS for analysis of essential oils from clove buds. *Sustainable Chemistry and Pharmacy,* 27: 100695. Available: https://www.sciencedirect.com/science/article/pii/S2352554122000997 (Accessed 8 February 2023).

Chintalchere, J. M., Dar, M. A., Raut, K. D. and Pandit, R. S. 2021. Bioefficacy of lemongrass and tea tree essential oils against house fly, Musca domestica. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences,* 91: 307-318. Available: https://link.springer.com/article/10.1007/s40011-020-01220-z (Accessed 23 February 2023).

Cimino, C., Maurel, O. M., Musumeci, T., Bonaccorso, A., Drago, F., Souto, E. M. B., Pignatello, R. and Carbone, C. 2021. Essential Oils: Pharmaceutical Applications and Encapsulation Strategies into Lipid-Based Delivery Systems. *Pharmaceutics,* 13 (3): 327. Available: https://www.mdpi.com/1999-4923/13/3/327 (Accessed 27 July 2022).

Clark, A. M., Magawa, C., Pliego-Zamora, A., Low, P., Reynolds, M. and Ralph, S. J. 2021. Tea tree oil extract causes mitochondrial superoxide production and apoptosis as an anticancer agent, promoting tumor infiltrating neutrophils cytotoxic for breast cancer to induce tumor regression. *Biomedicine & Pharmacotherapy,* 140: 111790. Available: https://www.sciencedirect.com/science/article/pii/S0753332221005722 (Accessed 2 February 2022).

Collins Dictionaries. 2018. Glasgow: HarperCollins Publisher. Available: https://www.collinsdictionary.com/dictionary/english/extract (Accessed 19 October 2021).

Conde-Hernández, L. A., Espinosa-Victoria, J. R., Trejo, A. and Guerrero-Beltrán, J. Á. 2017. CO2-supercritical extraction, hydrodistillation and steam distillation of essential oil of rosemary (Rosmarinus officinalis). *Journal of food engineering,* 200: 81-86. Available: https://doi.org/10.1016/j.jfoodeng.2016.12.022 (Accessed 19 April 2022).

Cordeiro, L., Figueiredo, P., Souza, H., Sousa, A., Andrade-Júnior, F., Medeiros, D., Nóbrega, J., Silva, D., Martins, E. and Barbosa-Filho, J. 2020. Terpinen-4-ol as an Antibacterial and Antibiofilm Agent against Staphylococcus aureus. *International Journal of Molecular Sciences,* 21 (12): 4531. Available: https://www.mdpi.com/1422-0067/21/12/4531 (Accessed 19 February 2023).

da Silva, W. M. F., Kringel, D. H., de Souza, E. J. D., da Rosa Zavareze, E. and Dias, A. R. G. 2022. Basil essential oil: Methods of extraction, chemical composition, biological activities, and food applications. *Food and Bioprocess Technology,* 15 (1): 1-27. Available: https://link.springer.com/article/10.1007/s11947-021-02690-3 (Accessed 14 February 2023).

Danthu, P., Simanjuntak, R., Fawbush, F., Leong, J., Tsy, J.-M., Abdillahi, M. M., Jahiel, M. and Penot, E. 2021. The clove tree and its products (clove bud, clove oil, eugenol): prosperous today but what of tomorrow's restrictions? *International Journal of Tropical and Subtropical Horticulture* 75 (5): 224-242. Available: https://www.researchgate.net/publication/348649211_The_clove_tree_and_its_products_clove_bud_clove_oil_eugenol_prosperous_today_but_what_of_tomorrow%27s_restrictions (Accessed 30 April 2022).

Daverey, A. and Dutta, K. 2021. COVID-19: Eco-friendly hand hygiene for human and environmental safety. *Journal of environmental chemical engineering,* 9 (2): 104754. Available: https://doi.org/10.1016/j.jece.2020.104754 (Accessed 18 August 2022).

DeCarlo, M. 2018. *Scientific Inquiry in Social Work. Mountain View: Pressbooks.* Available: https://scientificinquiryinsocialwork.pressbooks.com/chapter/12-1-experimental-design-what-is-it-and-when-should-it-be-used (Accessed 22 February 2020).

Dehdashtian, A., Stringer, T. P., Warren, A. J., Mu, E. W., Amirlak, B. and Shahabi, L. 2018. *Anatomy and physiology of the skin* Springer. Available: https://link.springer.com/chapter/10.1007/978-3-319-78310-9_2 (Accessed 29 August 2022).

Di Consiglio, M., Sturabotti, E., Brugnoli, B., Piozzi, A., Migneco, L. M. and Francolini, I. 2023. Synthesis of sustainable eugenol/hydroxyethylmethacrylate-based polymers with antioxidant and antimicrobial properties. *Polymer Chemistry,* Article ID. Available: https://pubs.rsc.org/en/content/articlelanding/2023/py/d2py01183b/unauth (Accessed 19 July 2023).

E.G Hecht, T. J. C. 2020. *Isopropyl Alcohol-Benzocaine 70%-6% Topical Pads Side Effects by Likelihood and Severity*. Available: https://www.webmd.com/drugs/2/drug-149330/isopropyl-alcohol-benzocaine-topical/details/list-sideeffects (Accessed 30 September 2020).

Elshafie, H. S. and Camele, I. 2017. An overview of the biological effects of some mediterranean essential oils on human health. *BioMed research international*, 2017. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5694587/#__sec2title

(Accessed 10 March 2021).

Ercioglu, E., Velioglu, H. M. and Boyaci, I. H. 2018. Determination of terpenoid contents of aromatic plants using NIRS. *Talanta*, 178: 716-721. Available: https://pubmed.ncbi.nlm.nih.gov/29136886/ (Accessed 31 August 2022).

Fekri, N., El Amir, D., Owis, A. and AbouZid, S. 2021. Studies on essential oil from rose-scented geranium, Pelargonium graveolens L'Hérit. (Geraniaceae). *Natural Product Research*, 35 (15): 2593-2597. Available: 10.1080/14786419.2019.1682581 (Accessed 29 August 2022).

Freitas, J. V. B., Alves Filho, E. G., Silva, L. M. A., Zocolo, G. J., de Brito, E. S. and Gramosa, N. V. 2018. Chemometric analysis of NMR and GC datasets for chemotype characterization of essential oils from different species of Ocimum. *Talanta*, 180: 329-336. Available: https://doi.org/10.1016/j.talanta.2017.12.053 (Accessed 29 August 2022).

Galovičová, L., Borotová, P., Valková, V., Vukovic, N. L., Vukic, M., Štefániková, J., Ďúranová, H., Kowalczewski, P. Ł., Čmiková, N. and Kačániová, M. 2021. Thymus vulgaris Essential Oil and Its Biological Activity. *Plants*, 10 (9): 1959. Available: https://www.mdpi.com/2223-7747/10/9/1959 (Accessed 25 July 2022).

Godlewska, K., Ronga, D. and Michalak, I. 2021. Plant extracts-importance in sustainable agriculture. *Italian Journal of Agronomy*, 16 (2). Available: https://agronomy.it/index.php/agro/article/view/1851/1296 (Accessed 27 July 2022).

Griffin, S. G., Markham, J. L. and Leach, D. N. 2000. An agar dilution method for the determination of the minimum inhibitory concentration of essential oils. *Journal of Essential Oil Research*, 12 (2): 249-255. Available: https://www.tandfonline.com/doi/abs/10.1080/10412905.2000.9699509 (Accessed 19 July 2023).

Guzmán, E. and Lucia, A. 2021. Essential oils and their individual components in cosmetic products. *Molecules*, 8 (4): 114. Available: https://www.mdpi.com/2079-9284/8/4/114https://www.mdpi.com/1420-3049/26/3/666 (Accessed 25 July 2022).

Halahlah, A., Kavetsou, E., Pitterou, I., Grigorakis, S., Loupassaki, S., Tziveleka, L.-A., Kikionis, S., Ioannou, E. and Detsi, A. 2021. Synthesis and characterization of inclusion complexes of rosemary essential oil with various β-cyclodextrins and evaluation of their antibacterial activity against Staphylococcus aureus. *Journal of Drug Delivery Science and Technology*, 65: 102660. Available: https://www.sciencedirect.com/science/article/pii/S1773224721003403 (Accessed 29 August 2022).

Hani Yousef; Mandy Alhajj; Sandeep Sharma. 2021. *Anatomy, Skin (Integument), Epidermis* Available: https://www.ncbi.nlm.nih.gov/books/NBK470464/ (Accessed 19 February 2022).

Haro-González, J. N., Castillo-Herrera, G. A., Martínez-Velázquez, M. and Espinosa-Andrews, H. 2021. Clove essential oil (Syzygium aromaticum L. Myrtaceae): Extraction, chemical composition, food applications, and essential bioactivity for human health. *Molecules*, 26 (21): 6387. Available: https://www.mdpi.com/1420-3049/26/21/6387 (Accessed 20 February 2023).

Hartatie, E., Prihartini, I., Widodo, W. and Wahyudi, A. 2019. Bioactive Compounds of Lemongrass (Cymbopogon citratus) essential oil from different parts of the plant and distillation methods as natural antioxidant in broiler meat. In: Proceedings of *IOP Conference Series: Materials Science and Engineering*. IOP Publishing, 012018. Available: https://iopscience.iop.org/article/10.1088/1757-899X/532/1/012018/pdf (Accessed 10 May 2022).

Hassine, D. B., Euch, S. K. E., Rami, R., Nessrine, G., Rouguiata, K., Manef, A. and Jalloul, B. 2021. Clove buds essential oil: the impact of grinding on the chemical composition and its biological activities involved in consumer's health security. *BioMed research international*, 2021. Available: https://www.hindawi.com/journals/bmri/2021/9940591/ (Accessed 14 July 2022).

Her, J. and Cho, M.-K. 2021. Effect of aromatherapy on sleep quality of adults and elderly people: A systematic literature review and meta-analysis. *Science Direct*, 60: 102739. Available: https://www.sciencedirect.com/science/article/pii/S0965229921000807 (Accessed 27 July 2022).

Herman, R. A., Ayepa, E., Shittu, S., Fometu, S. S. and Wang, J. 2019. Essential oils and their applications-a mini review. *Adv. Nutr. Food Sci*, 4 (4). Available: https://www.researchgate.net/profile/Richard-Herman-5/publication/336305801_Essential_Oils_and_their_applications-A_mini_review/links/5e92a663299bf1307991511f/Essential-Oils-and-their-applications-A-mini-review.pdf (Accessed 14 July 2022).

Hoffman, M. 2021. *Human Anatomy*. Available: https://www.webmd.com/skin-problems-and-treatments/picture-of-the-skin (Accessed 28/01/2022).

Hossain, M. L., Lim, L. Y., Hammer, K., Hettiarachchi, D. and Locher, C. 2022. A Review of Commonly Used Methodologies for Assessing the Antibacterial Activity of Honey and Honey

Products. *Antibiotics*, 11 (7): 975. Available: mdpi.com/2079-6382/11/7/975 (Accessed 19 February 2023

).

Johnson, C., Eix, E., Lam, B., Wartman, K., Meudt, J., Shanmuganayagam, D. and Nett, J. 2021. *Augmenting the Activity of Chlorhexidine for Decolonization of Candida auris from Porcine skin. J. Fungi 2021, 7, 804*: s Note: MDPI stays neutral with regard to jurisdictional claims in published …. Available: https://d1wqtxts1xzle7.cloudfront.net/81530577/pdf-libre.pdf?1646168423=&response-content-disposition=inline%3B+filename%3DAugmenting_the_Activity_of_Chlorhexidine.pdf&Expires=1657610738&Signature=Z2s-7amiD18uid6HxEPfCPASoi6uahmF-lfcDeJfZrwYycl-1a96VQjpTDAswc5OjBCfv0GrBt4SsboP4tBRVBv5bbkmg3QGpSfFelVkkbJDa6WYMpw90syNKrzZJE1OBZwQPtYsvXGUt35hNH~8BAGru~r0AfEFQyparhx5Am1IWODIoPLr9urxaUxXHdl--PHPunum9qAqQ~VFle-NksDZz3gLN34ZLjyavU1jJQPNNTZkyMi10aulpwDXNDuPmQB-X0bedR63GP2BJaVHEybLvuhKyAGq6OXWUeUZdhNRcl6IXtf7tCnHpfv3Rx3htbSLeWUq4iHMb1LKJPsZNQ__&Key-Pair-Id=APKAJLOHF5GGSLRBV4ZA (Accessed 12 July 2022).

Ju, J., Xie, Y., Yu, H., Guo, Y., Cheng, Y., Qian, H. and Yao, W. 2022. Synergistic interactions of plant essential oils with antimicrobial agents: a new antimicrobial therapy. *Critical Reviews in Food Science and Nutrition*, 62 (7): 1740-1751. Available: https://www.tandfonline.com/doi/full/10.1080/10408398.2020.1846494?casa_token=L-t10W1NDTQAAAAA%3AlLWLKo4zAUi_jF_bj16GzF3xZ6XXrmbz2MEsX9KXDHo_LKT_p5Pir9xTpIpVfHfm-GOMUAIhVOIK4eU (Accessed 27 July 2022).

Kapelle, I. B. D., Souhoka, F. A. and Walla, A. M. Indonesian Journal of Chemical Research. Article ID. Available: https://d1wqtxts1xzle7.cloudfront.net/89409623/4159-libre.pdf?1660025481=&response-content-disposition=inline%3B+filename%3DChemical_Composition_Oil_and_Ethanol_Ext.pdf&Expires=1676367758&Signature=HKOoEPKMJRpyI~qGw7QNAQZmt5Y9153ytXYUiAg1z5Th-2qHjVX3CfxD5oJTZ0T1j7rKQFmmjDQMG5cl3b3k39ueljImFirLnp3sHYbgJvkAJjnWYM0gRiGQ7VUGeUlCugPn9xgWG5797Bop4S7GMLwFrnVCUx~T4kJQ1dWpOySJuYt~5BNEO85EqZmoS0Z59tIiwg6aPkWR86tkDhahYFtwhBoLIuGkGpi61IDXGtY0b3LllqgSiJfHgJhXJ0GrOwlXZNz-UD9859aqj15OPUecp4h5JOn7Iz1YM7BCOKoqaX4cFm3xw8mznOdwjwkxto-SFdskZBaBlM8bea6KHw__&Key-Pair-Id=APKAJLOHF5GGSLRBV4ZA (Accessed 14 February 2023).

Kim, M., Chhoeun, T. B., Kim, T., Sowndhararajan, K. and Kim, S. 2020. The gender variation on the electroencephalographic activity in response to the exposure of black pepper essential oil from Kampong Cham, Cambodia. *Flavour and fragrance journal*, 35 (3): 284-293. Available: https://onlinelibrary.wiley.com/doi/full/10.1002/ffj.3560?casa_token=nz8dwkwK1Z4AAAAA%3AsJiAy5Z3K6Cgp69ut0S7lKaW1UnR21Iz1jPtoA0w9ZkC-QaGUjrf40enWycNpniN1_m_I2wVC1jX9HVm (Accessed 19 February 2023).

Kumoro, A., Wardhani, D., Retnowati, D. and Haryani, K. 2021. A brief review on the characteristics, extraction and potential industrial applications of citronella grass (Cymbopogon nardus) and lemongrass (Cymbopogon citratus) essential oils. *IOP Conference Series: Materials Science and Engineering,* 1053 (1): 012118. Available: https://iopscience.iop.org/article/10.1088/1757-899X/1053/1/012118/meta (Accessed 14 May 2022).

Lai-Cheong, J. E. and McGrath, J. A. 2017. Structure and function of skin, hair and nails. *Medicine,* 45 (6): 347-351. Available: https://kclpure.kcl.ac.uk/portal/en/publications/structure-and-function-of-skin-hair-and-nails(56453446-6b70-46d4-b979-4de18d657c24)/export.html (Accessed 29 August 2022).

Li, X., Shen, D., Zang, Q., Qiu, Y. and Yang, X. 2021. Chemical Components and Antimicrobial Activities of Tea Tree Hydrosol and Their Correlation With Tea Tree Oil. *Natural Product Communications,* 16 (9): 1934578X211038390. Available: 10.1177/1934578X211038390 (Accessed 14 October 2021).

Liu, L., Wang, Q., Lin, J., Owens, G. and Chen, Z. 2022. Enhanced 17α-estradiol removal by biosynthesized rGO@ Fe NPs using a response surface methodology. *Process Safety and Environmental Protection,* 159: 53-60. Available: https://www.sciencedirect.com/science/article/pii/S095758202100728X (Accessed 9 February 2023).

Liu, Y., Wang, Y., Xia, Z., Wang, Y., Wu, Y. and Gong, Z. 2019. Rapid determination of phytosterols by NIRS and chemometric methods. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy,* 211: 336-341. Available: https://doi.org/10.1016/j.saa.2018.12.030 (Accessed 31 August 2022).

Loose, M., Pilger, E. and Wagenlehner, F. 2020. Anti-Bacterial Effects of Essential Oils against Uropathogenic Bacteria. *Antibiotics,* 9 (6): 358. Available: https://www.mdpi.com/2079-6382/9/6/358 (Accessed 19 July 2023).

Mahanta, B. P., Sut, D., Lal, M. and Haldar, S. 2021. Hydrodistillation alters the compositional originality in black turmeric (Curcuma caesia Roxb.) essential oil. *Journal of Essential Oil Research,* 33 (3): 240-246. Available: 10.1080/10412905.2021.1873868 (Accessed 29 August 2022).

Mahmoud, N., Abdou, M. A. H., Salaheldin, S. and Soliman, W. S. 2022. Lemongrass Growth, Essential Oil, and Active Substances as Affected by Water Deficit. *Horticulturae,* 8 (3): 250. Available: https://www.mdpi.com/2311-7524/8/3/250 (Accessed 28 February 2023).

Malabadi, R. B., Kolkar, K. P., Meti, N. T. and Chalannavar, R. K. 2021. Role of plant based hand sanitizers during the recent outbreak of coronavirus (SARS-CoV-2) disease (Covid-19). Article ID. Available: https://www.researchgate.net/publication/353018615_Role_of_Plant_Based_Hand_Sanitizers_During_the_Recent_Outbreak_of_Coronavirus_SARS-CoV-2_Disease_Covid-19 (Accessed 29 August 2022).

Manojkumar, N., Muthukumaran, C. and Sharmila, G. 2022. A comprehensive review on the application of response surface methodology for optimization of biodiesel production using different oil sources. *Journal of King Saud University-Engineering Sciences*, 34 (3): 198-208. Available: https://www.sciencedirect.com/science/article/pii/S1018363920303123 (Accessed 9 February 2023).

Maurya, A., Prasad, J., Das, S. and Dwivedy, A. K. 2021. Essential oils and their application in food safety. *Frontiers in Sustainable Food Systems*, 5: 653420. Available: https://www.mdpi.com/2304-8158/11/3/464 (Accessed 25 July 2022).

Meilina, H. and Munawar, A. 2021. Robust fraudulence detection of patchouli oil plant using near infrared spectroscopy. In: Proceedings of *IOP Conference Series: Earth and Environmental Science*. IOP Publishing, 012019. Available: https://iopscience.iop.org/article/10.1088/1755-1315/644/1/012019 (Accessed 31 August 2022).

Michalczyk, A. and Ostrowska, P. 2021. Essential oils and their components in combating fungal pathogens of animal and human skin. *Journal of Medical Mycology*, 31 (2): 101118. Available: https://www.sciencedirect.com/science/article/pii/S1156523321000081 (Accessed 25 July 2022).

Mori, Natsuki and Toyonobu. 2022. Extraction of essential oils from tea tree (Melaleuca alternifolia) and lemon grass (Cymbopogon citratus) using betaine‐based deep eutectic solvent (DES). *Phytochemical Analysis*, 33 (6): 831-837. Available: https://analyticalsciencejournals.onlinelibrary.wiley.com/doi/full/10.1002/pca.3132 (Accessed 8 February 2023).

Mukarram, M., Khan, M. M. A., Zehra, A., Choudhary, S., Naeem, M. and Aftab, T. 2021. Biosynthesis of Lemongrass Essential Oil and the Underlying Mechanism for Its Insecticidal Activity. *Medicinal and Aromatic Plants: Healthcare and Industrial Applications*, Article ID: 429-443. Available: 10.1007/978-3-030-58975-2_18 (Accessed 15 May 2022).

Muta, T., Parikh, A., Kathawala, K., Haidari, H., Song, Y., Thomas, J. and Garg., S. 2020. Quality-by-Design Approach for the Development of Nano-Sized Tea Tree Oil Formulation-Impregnated Biocompatible Gel with Antimicrobial Properties

Pharmaceutics, 12 (11): 1091. Available: https://www.mdpi.com/1999-4923/12/11/1091/htm#B7-pharmaceutics-12-01091 (Accessed 9 May 2022).

Nair, A., Mallya, R., Suvarna, V., Khan, T. A., Momin, M. and Omri, A. 2022. Nanoparticles—Attractive carriers of antimicrobial essential oils. *Antibiotics*, 11 (1): 108. Available: https://www.mdpi.com/2079-6382/11/1/108 (Accessed 9 February 2023).

Nazir, I. and Gangoo, S. A. 2022. Pharmaceutical and Therapeutic Potentials of Essential Oils. In: *Essential Oils-Advances in Extractions and Biological Applications*. IntechOpen.

Nyiew, K. Y., Kwong, P. J. and Yow, Y. Y. 2022. An overview of antimicrobial properties of kombucha. *Comprehensive Reviews in Food Science and Food Safety*, 21 (2): 1024-1053. Available: https://ift.onlinelibrary.wiley.com/doi/full/10.1111/1541-4337.12892?casa_token=65rDBbpG6LwAAAAA%3AWvfwfIWkG959Ihadtzu8P_K_Ic73ZfN2qgmq6hq0jFrpNtZq1cTAqA1QsQWGN2ToACZSgD7Jh86FwH1N (Accessed 19 February 2023).

Ogundajo, A. L., Ewekeye, T., Sharaibi, O. J., Owolabi, M. S., Dosoky, N. S. and Setzer, W. N. 2021. Antimicrobial activities of sesquiterpene-rich essential oils of two medicinal plants, Lannea egregia and Emilia sonchifolia, from Nigeria. *Plants*, 10 (3): 488. Available: https://www.mdpi.com/2223-7747/10/3/488 (Accessed 19 February 2023).

Pathirana, H., Wimalasena, S., De Silva, B., Hossain, S. and Heo, G. 2019. Antibacterial activity of clove essential oil and eugenol against fish pathogenic bacteria isolated from cultured olive flounder (Paralichthys olivaceus). Slov. *Vet. Res*, 56: 31-38. Available: https://www.researchgate.net/profile/Gang-Joon-Heo/publication/331915343_Antibacterial_activity_of_clove_essential_oil_and_eugenol_against_fish_pathogenic_bacteria_isolated_from_cultured_olive_flounder_Paralichthys_olivaceus/links/5e5722e3299bf1bdb83e5ee2/Antibacterial-activity-of-clove-essential-oil-and-eugenol-against-fish-pathogenic-bacteria-isolated-from-cultured-olive-flounder-Paralichthys-olivaceus.pdf (Accessed 19 July 2023).

Pedrós-Garrido, S., Clemente, I., Calanche, J., Condón-Abanto, S., Beltrán, J., Lyng, J., Brunton, N., Bolton, D. and Whyte, P. 2020. Antimicrobial activity of natural compounds against Listeria spp. and their effects on sensory attributes in salmon (Salmo salar) and cod (Gadus morhua). *Food Control*, 107: 106768. Available: https://www.sciencedirect.com/science/article/pii/S0956713519303573 (Accessed 19 July 2023).

Ponen, S. 2020. *Antibiotic resistance - prevention*. Available: https://www.healthnavigator.org.nz/medicines/a/antibiotic-resistance-prevention/ (Accessed 18 August 2022).

Purba, R. A. P. and Paengkoum, P. 2019. Bioanalytical HPLC method of Piper betle L. for quantifying phenolic compound, water-soluble vitamin, and essential oil in five different solvent extracts. *Journal of Applied Pharmaceutical Science,* 9 (5): 33-39. Available: https://www.researchgate.net/publication/333090198_Bioanalytical_HPLC_method_of_Piper_Betle_L_for_quantifying_phenolic_compound_water-soluble_vitamin_and_essential_oil_in_five_different_solvent_extracts (Accessed 30 August 2022).

Rahmi, D., Yunilawati, R., Jati, B. N., Setiawati, I., Riyanto, A., Batubara, I. and Astuti, R. I. 2021. Antiaging and Skin Irritation Potential of Four Main Indonesian Essential Oils. *Cosmetics,* 8 (4): 94. Available: https://www.mdpi.com/2079-9284/8/4/94/htm (Accessed 27 July 2022).

Recio-Cázares, S. L., López-Malo, A., Ramírez-Corona, N. and Palou, E. 2023. Relationship Between the Chemical Composition and

Transport Properties with the Antimicrobial Activity of

Essential Oil from Leaves of Mexican Lippia (Aloysia

citriodora) Extracted by Hydro-Distillation. *Biointerface Research in Applied Chemistry,* 13 (6). Available: https://biointerfaceresearch.com/wp-content/uploads/2023/02/BRIAC136.560.pdf (Accessed 25 March 2023).

Rojas, J., Cabrera, S., Benavides, J., Lopera, Y. and Yarce, C. J. 2021. Lipidic Matrixes Containing Clove Essential Oil: Biological Activity, Microstructural and Textural Studies. *Molecules,* 26 (9): 2425. Available: https://www.mdpi.com/1420-3049/26/9/2425 (Accessed 28 August 2022).

Sabaoui, Z. and Lakhdar, L. 2021. Essential Oils in Periodontics. What is the Interest? *Integrative Journal of Medical Sciences,* 8. Available: https://www.mbmjpress.com/index.php/ijms/article/view/499 (Accessed 14 February 2023).

Salvino, R. A., Aroulanda, C., De Filpo, G., Celebre, G. and De Luca, G. 2022. Metabolic composition and authenticity evaluation of bergamot essential oil assessed by nuclear magnetic resonance spectroscopy. *Analytical and Bioanalytical Chemistry,* 414 (6): 2297-2313. Available: 10.1007/s00216-021-03869-5 (Accessed 29 August 2022).

Saroj, A., Oriyomi, O. V., Nayak, A. K. and Haider, S. Z. 2020. Phytochemicals of plant-derived essential oils: A novel green approach against pests. *Natural remedies for pest, disease and weed control,* Article ID: 65-79. Available: https://www.mdpi.com/2076-3417/10/22/8103 (Accessed 19 February 2023).

Schripsema, J., da Silva, S. M. and Dagnino, D. 2022. Differential NMR and chromatography for the detection and analysis of adulteration of vetiver essential oils. *Talanta,* 237: 122928. Available: https://doi.org/10.1016/j.talanta.2021.122928 (Accessed 18 February 2022).

Sharmeen, J. B., Mahomoodally, F. M., Zengin, G. and Maggi, F. 2021. Essential Oils as Natural Sources of Fragrance Compounds for Cosmetics and Cosmeceuticals. *Molecules*, 26 (3): 666. Available: https://www.mdpi.com/1420-3049/26/3/666 (Accessed 23 August 2022).

Shiraly, R., Askarian, M., Longtin, Y. and Nematian, S. S. S. 2021. Alcohol-containing Hand Rubs: A Survey on Beliefs and Self-reported Practices of Muslim Nurses. *Africa Journal of Nursing and Midwifery*, 23 (2): 15 pages-15 pages. Available: https://journals.co.za/doi/pdf/10.25159/2520-5293/8927 (Accessed 18 August 2022).

Singh, S., Singh, B., Kumar, K., Rani, P., Kumar, T. and Arya, A. M. 2018. Medico-Nutritional facts of lemongrass (Cymbopogon citrates): A review. *Annals of Horticulture*, 11 (2): 186-194. Available: https://indianjournals.com/ijor.aspx?target=ijor:ah&volume=11&issue=2&article=013 (Accessed 11 May 2022).

Sirichan, T., Kijpatanasilp, I., Asadatorn, N. and Assatarakul, K. 2022. Optimization of ultrasound extraction of functional compound from makiang seed by response surface methodology and antimicrobial activity of optimized extract with its application in orange juice. *Ultrasonics Sonochemistry*, 83: 105916. Available: https://www.sciencedirect.com/science/article/pii/S1350417722000098 (Accessed 9 February 2023).

Siroua, K., El Ghallab, Y., Mouss, R. A., Kadiri, F., Belamine, H., El Kouali, M. and Kenz, A. 2022. Chemical composition of essential oil from invasive Moroccan Cyperus rotundus L., in vitro antimicrobial and antiradical activities, and in silico molecular docking of major compounds on drug efflux pumps. *South African Journal of Botany*, 147: 782-789. Available: https://www.sciencedirect.com/science/article/pii/S0254629922001077?casa_token=-l_ygSYULq0AAAAA:Z6k0pY0jd79pIEZDHw-tch_nLZqOZasfiNgH8VDZb6DNVNCpdn6jqq6CzJxiXcpXnKR4uTY3X3A (Accessed 19 February 2023).

Soares, G. A. B. e., Bhattacharya, T., Chakrabarti, T., Tagde, P. and Cavalu, S. 2021. Exploring pharmacological mechanisms of essential oils on the central nervous system. *Plants*, 11 (1): 21. Available: https://pubmed.ncbi.nlm.nih.gov/35009027/ (Accessed 23 August 2022).

Sobhy, S., Al-Askar, A. A., Bakhiet, E. K., Elsharkawy, M. M., Arishi, A. A., Behiry, S. I. and Abdelkhalek, A. 2023. Phytochemical Characterization and Antifungal Efficacy of Camphor (Cinnamomum camphora L.) Extract against Phytopathogenic Fungi. *Separations*, 10 (3): 189. Available: https://www.mdpi.com/2297-8739/10/3/189 (Accessed 18 July 2023).

Spesyvyi, A., Španěl, P. and Sovová, K. 2019. Styrene radical cations for chemical ionization mass spectrometry analyses of monoterpene hydrocarbons. *Rapid Communications in Mass Spectrometry,* 33 (24): 1870-1876. Available: https://analyticalsciencejournals.onlinelibrary.wiley.com/doi/full/10.1002/rcm.8556?casa_token=chwkuPHEtkgAAAAA%3AcsvGcOtum8prQQ1NCGlHLc757Fd7w_c-qIX5sot96UiKpS8TnW3UyYY2dksjPRRSq--3Zn8IV_W0epJB (Accessed 23 February 2023).

Taalab, M. R., Mahmoud, S. A., Moslemany, R. M. E. and Abdelaziz, D. M. 2021. Intrapocket application of tea tree oil gel in the treatment of stage 2 periodontitis. *BMC Oral Health,* 21 (1): 239. Available: 10.1186/s12903-021-01588-y (Accessed 7 May 2021).

Tang, Y., Gong, M., Qin, X., Su, H., Wang, Z. and Dong, H. 2021. The Therapeutic Effect of Aromatherapy on Insomnia: a Meta-Analysis. *Journal of Affective Disorders,* 288: 1-9. Available: https://doi.org/10.1016/j.jad.2021.03.066 (Accessed 27 July 2022).

Torres Neto, L., Monteiro, M. L. G., Machado, M. A. M., Galvan, D. and Conte Junior, C. A. 2022. An Optimization of Oregano, Thyme, and Lemongrass Essential Oil Blend to Simultaneous Inactivation of Relevant Foodborne Pathogens by Simplex–Centroid Mixture Design. *Antibiotics,* 11 (11): 1572. Available: https://www.mdpi.com/2079-6382/11/11/1572 (Accessed 19 July 2023).

Trang, D. T., Hoang, T. K. V., Nguyen, T. T. M., Van Cuong, P., Dang, N. H., Dang, H. D., Nguyen Quang, T. and Dat, N. T. 2020. Essential oils of lemongrass (Cymbopogon citratus Stapf) induces apoptosis and cell cycle arrest in A549 lung cancer cells. *BioMed research international,* 2020. Available: https://www.hindawi.com/journals/bmri/2020/5924856/ (Accessed 19 February 2023).

Unalan, I. and Boccaccini, A. R. 2021. Essential oils in biomedical applications: Recent progress and future opportunities. *Current Opinion in Biomedical Engineering,* 17: 100261. Available: https://www.sciencedirect.com/science/article/pii/S2468451121000015 (Accessed 18 August 2022).

Unicef. 2020. *Fact Sheet: Lack of handwashing with soap puts millions at increase risk to COVID-19 and other infectios disease.* Available: https://www.unicef.org/press-releases/fact-sheet-lack-handwashing-soap-puts-millions-increased-risk-covid-19-and-other (Accessed 12 June 2022).

Van, H. T., Do, T. T., Luu, T. N., Le, V. S. and Doan, V. D. 2021. Chemical constituents and antibacterial activities of essential oils from aerial parts of three Limnophila species (Scrophulariaceae) in Southern Vietnam. *Research Journal of Chemistry and Environment,* 25 (4): 12-19. Available: https://www.researchgate.net/profile/Thien-Van-2/publication/355109134_Chemical_constituents_and_antibacterial_activities_of_essential_oils_from_aerial_parts_of_three_Limnophila_species_Scrophulariaceae_in_Southern_Vietnam/links/

615e665cc04f5909fd89f1e2/Chemical-constituents-and-antibacterial-activities-of-essential-oils-from-aerial-parts-of-three-Limnophila-species-Scrophulariaceae-in-Southern-Vietnam.pdf (Accessed 14 February 2023).

Vermeil, T. P., A.; Kilpatrick,C.; Pires,D., Allergranzi,B.; Pittet,D. 2019. Hand hygiene in hospitals: anatomy of a revolution. *Journal of Hospital infection*, 101 (4): 383-392. Available: https://www.sciencedirect.com/science/article/pii/S0195670118304821?casa_token=vqSrHJxMQJsAAAAA:wAbc8hd6yOZRKh7_U3Y1ArO8mGSOxPDSAnZYuySNvdU8zeWF5-xVgBde1GWzTk5t2bAjPAFYyHgJ (Accessed 2 February 2021).

Wahyudi, D., Noor, E., Setyaningsih, D., Djatna, T. and Irmansyah, I. 2022. Prediction of rhodinol content in Java citronella oil using NIR spectroscopy in the initial stage developing a spectral smart sensor system. *Research in Agricultural Engineering*, Article ID. Available: https://www.agriculturejournals.cz/web/rae.htm?type=article&id=87_2021-RAE (Accessed 31 August 2022).

Wang, M., Zhao, J., Ali, Z., Avonto, C. and Khan, I. A. 2021. A novel approach for lavender essential oil authentication and quality assessment. *Journal of Pharmaceutical and Biomedical Analysis*, 199: 114050. Available: https://www.sciencedirect.com/science/article/pii/S073170852100162X (Accessed 29 August 2022).

Wang, Y., Sun, L., Wu, J. and Ma, C. 2018. Antioxidant Activity and Chemical Composition Analysis of Essential Oil from Cinnamomum longepaniculatum by NIRS and GC-MS. Article ID. Available: https://www.researchgate.net/publication/324695692_Antioxidant_Activity_and_Chemical_Composition_Analysis_of_Essential_Oil_from_Cinnamomum_longepaniculatum_by_NIRS_and_GC-MS (Accessed 31 August 2022).

Wania, A. R. Y., Kanchan Khursheed, Aadil Rather, Manzoor Ahmad 2021. An updated and comprehensive review of the antiviral potential of essential oils and their chemical constituents with special focus on their mechanism of action against various influenza and coronaviruses. *Microbial Pathogenesis*, 152 (104620). Available: 10.1016/j.micpath.2020.104620 (Accessed 19 October 2021).

Williams, Y. 2020. *True Experiment: Definitions & Examples* Available: https://study.com/academy/lesson/true-experiment-definition-examples.html (Accessed 23 February 2021).

World Health Organisation. 2019. *Coronavirus disease (Covid-19)*. Available: https://www.who.int/emergencies/diseases/novel-coronavirus-2019/advice-for-public/coronavirus-disease-covid-19 (Accessed 18 August 2022).

Xiao, S., Cui, P., Shi, W. and Zhang, Y. 2019. Identification of essential oils with strong activity against stationary phase uropathogenic Escherichia coli. *bioRxiv,* Article ID: 702951. Available: https://www.biorxiv.org/content/10.1101/702951v1.abstract (Accessed 19 July 2023).

Yasin, M., Younis, A., Javed, T., Akram, A., Ahsan, M., Shabbir, R., Ali, M. M., Tahir, A., El-Ballat, E. M. and Sheteiwy, M. S. 2021. River Tea Tree Oil: Composition, Antimicrobial and Antioxidant Activities, and Potential Applications in Agriculture. *Plants*, 10 (10): 2105. Available: https://www.mdpi.com/2223-7747/10/10/2105/htm (Accessed 28 July 2022).

Yu, W. 2021. Applications of near infrared spectroscopy for fish and fish products quality: a review. In: Proceedings of *IOP Conference Series: Earth and Environmental Science*. IOP Publishing, 012115. Available: https://iopscience.iop.org/article/10.1088/1755-1315/657/1/012115/pdf (Accessed 31 August 2022).

Zia, K., Siddiqui, T., Ali, S., Farooq, I., Zafar, M. S. and Khurshid, Z. 2019. Nuclear Magnetic Resonance Spectroscopy for Medical and Dental Applications: A Comprehensive Review. *Eur J Dent*, 13 (01): 124-128. Available: https://www.mdpi.com/2076-3417/10/20/7294 (Accessed 29 August 2022).

ANNEXURE:1

escentia

"Your well-being, in your hands"

CERTIFICATE OF ANALYSIS

Product:	- Clove Bud Organic Essential Oil
INCI Name:	- *Eugenia caryophyllata*
Batch Number:	- ESC10156O
Manufacturing Date:	- 06/2021
Retest Date:	- 06/2026
Country of Origin:	- MADAGASCAR

CHEMICAL AND PHYSICAL DESCRIPTION

Chemical Description				
Description	**Min Value**	**Max Value**	**Result**	**Compliance**
Specific Gravity @ 20°C	1.040	1.064	1.0592	Pass
Refractive Index @ 20°C	1.523	1.535	1.5328	Pass
Optical Rotation	0°	-1.50°	-0.52°	Pass
Physical Description				
Appearance		Liquid		Pass
Colour		Colourless to Pale Yellow		Pass
Odour		Spicy Aroma		Pass
Major Constituent Listing:				
Name of Constituents	**Specifications (%)**		**% Compositions**	
	Min Value	**Max Value**		
Anethole	-	-	0.268	
Eugenol	70	90	80.761	
Caryophyllene	5	12	6.042	

Alpha Humulene	-	-	0.733
Eugenyl Acetate	8	17	11.546
Caryophyllene Oxide	-	-	0.392

This information is believed to be current and accurate, but is provided without any warranty expressed or implied.
This Certificate of Analysis has been produced electronically and is valid without a signature.

ANNEXURE:2

escentia

"Your well-being, in your hands"

CERTIFICATE OF ANALYSIS

Product:	- Tea Tree Organic Essential Oil
INCI Name:	- *Melaleuca alternifolia*
Batch Number:	- ESC101980
Manufacturing Date:	- 05/2021
Retest Date:	- 05/2025
Country of Origin:	- SOUTH AFRICA

CHEMICAL AND PHYSICAL DESCRIPTION

Chemical Description				
Description	**Min Value**	**Max Value**	**Result**	**Compliance**
Specific Gravity @ 20°C	0.880	0.904	0.894	Pass
Refractive Index @ 20°C	1.476	1.482	1.477	Pass
Optical Rotation	+7°	+12°	+9.89°	Pass
Physical Description				
Appearance		Mobile Liquid		Pass
Colour		Colourless to Pale Yellow		Pass
Odour		Characteristic		Pass

This information is believed to be current and accurate, but is provided without any warranty expressed or implied.
This Certificate of Analysis has been produced electronically and is valid without a signature.

ESCENTIA PRODUCTS (PTY) LTD – 2018/445186/07 (Established 1994) - VAT Reg No. 4830180362
Unit E1, Fair-Oue Business Park, 19 Goud St, Benoni, Johannesburg, South Africa - Tel: (011) 425 – 3281
https://essential-oils.co.za/

ANNEXURE:3

ESCENTIA PRODUCTS (PTY) LTD – 2019/445189/07
(Established 1994)
VAT Reg No. 4830160362

escentia

Unit E1, Fair-Que Business Park, 19 Goud St, Benoni
(011) 425 – 3281
Directors: R P Aiken & M B Aiken

CERTIFICATE OF ANALYSIS

Product	Organic Lemongrass Essential Oil
Latin Name	Cymbopogon Flexuosus Oil
CAS No.	8007-02-1
Batch No.	ESC10991Q
Manufacture Date	October 2021
Retest Date	October 2026
Part of Plant Used	Grass (steam distillation)
Country of Origin	India
Storage Conditions	Store in a cool dark place

Physical Analysis	Specification		Results	
Colour	Clear Pale Yellow to Yellow		Complies	
Odour	Citrus Aroma		Complies	
Appearance	Liquid Oil		Complies	
Chemical Analysis	**Min Value**	**Max Value**	**Result**	**Conforms**
Refractive Index @ 20°C	1.4830	1.4890	1.4842	Pass
Relative Density @ 20°C	0.869	0.899	0.8954	Pass
Optical Rotation	- 3 °	+ 1°	- 1.76 °	Pass

Major Constituent Listing		
Name	**Results %**	**Specification %**
Camphene	0.924	-
Sulcatone (6-Methyl-5 Hepten-2-One)	1.301	-
Myrcene	-	0 - 12
Limonene	0.261	-
4 Nonanone	0.923	-
Linalool	1.052	0.4 - 2.0
Terpinen-4-OL	-	0.04 - 2.5
Alpha Terpineol	-	0 - 0.5
Decanal	0.120	-

ESCENTIA PRODUCTS (PTY) LTD – 2019/445189/07
(Established 1994)
VAT Reg No. 4930180362

escentia

Unit E1, Fair-Que Business Park, 19 Coud St, Benoni
(011) 425 – 3281
Directors: R P Aiken & M B Aiken

Major Constituent Listing		
Name	**Results %**	**Specification %**
Citronellol	0.132	-
Iso Neral	0.840	-
Iso Geranial	1.154	-
Neral	32.729	Citral > 70%
Geranial	41.554	
Geraniol	7.191	0.5 - 10
Citronellyl Acetate	-	-
Neryl Acetate	-	-
Geranyl Acetate	4.093	0.1 - 6
Beta Caryophyllene	2.017	0.09 - 1.2
Alpha Humulene	0.229	-
Gamma Cadinene	1.464	-
Caryophyllene Oxide	0.453	-

ANNEXURE:4 **The results for Lemongrass Organic oil Sample**

The concentration of the compounds is reported in terms of area percent (%)		
		Lemongrass Organic oil
Retention time (min)	Library identification	Area %
7,3814	Tricyclene	0,13
7,8711	alpha-Pinene	0,20
8,3794	CAMPHENE	1,00
10,0854	6-METHYL-5-HEPTEN-2-ONE	1,28
10,6295	N-Octanal	0,06
11,3639	l-LIMONENE	0,27
11,8434	TRANS-.BETA.-OCIMENE	0,26
12,1747	3,7-DIMETHYL-1,3,7-OCTATRIENE	0,17
13,0218	4-Nonanone	0,92
13,3975	.ALPHA.-TERPINOLENE	0,05
13,7972	Rosefuran	0,18
13,8994	Linalool	1,43
15,0197	5-Iminopyrrolidine-2-carbonitrile	0,15
15,3046	3,6-dimethyl-1,5-Heptadiene	0,26
15,3614	Photocitral A	0,33
15,5607	CITRONELLAL	0,29
15,8236	endo-Borneol	0,24

15,9227	1-tert-butyl-3,3-dimethylcyclopropene	0,82
16,2481	Rose furan epoxide	0,52
16,4766	Ethenylcyclohexane	1,30
16,6447	alpha-pipene	0,18
17,1425	N-DECANAL	0,18
17,915	Nerol	0,19
17,9938	L-CITRONELLOL	0,13
18,3402	NERAL	28,38
19,2584	GERANIAL	43,88
19,3593	trans-p-Mentha-1(7),8-dien-2-ol	0,19
21,4326	beta-bisabolene	0,50
21,4873	alpha-Longipinene	0,15
22,0971	Neryl acetate	5,39
22,1829	.BETA. ELEMENE	0,22
22,8514	.BETA.-CARYOPHYLLENE	2,84
23,6302	Isoeugenol	0,80
23,6858	ALPHA-HUMULENE	0,31
24,3763	GERMACRENE-B	0,26
24,7228	GERMACRENE-D	0,29
24,8747	alpha-Muurolene	0,11
24,9504	(1RS,2RS,1'SR)-1-(1-Methyloxyethyl)-2-vinylcyclobutane	0,10
25,2045	gamma-Cadinene	2,46
25,4304	.DELTA.-CADINENE	0,56
25,6345	CIS-.GAMMA.-BISABOLENE	0,44

26,0372	(E,Z)-.ALPHA.-FARNESENE	0,15
26,3601	GERANYL BUTYRATE	0,10
26,7856	CARYOPHYLLENE OXIDE	1,02
27,3873	spiro[4,4]nonan-2-one	0,11
36,863	FARNESOL 1	0,15
37,5042	FARNESOL 1	0,31
37,9996	3-(4,8-dimethyl-3,7-nonadienyl)-Furan	0,31

ANNEXURE: **The results for Clove Organic oil Sample** 5

The concentration of the compounds is reported in terms of area percent (%)		
		Clove Bud Organic oil
Retention time (min)	**Library identification**	**Area %**
16,6789	Methyl salicylate	0,35
18,5349	CHAVICOL	0,17
21,6477	EUGENOL	73,50
21,7876	ALPHA-COPAENE	0,12
22,3614	Vanillin	0,06
22,8899	BETA-CARYOPHYLLENE	5,72
23,699	ALPHA-HUMULENE	0,85
25,4241	DELTA-CADINENE	0,09
25,7337	EUGENYL ACETATE	17,38
26,4938	alpha-Caryophyllene alcohol	0,08
26,8048	CARYOPHYLLENE OXIDE	0,95
27,9199	(+)-trans-Isolimonene	0,06
28,0055	Adamantane	0,15
28,7814	(R)-(-)-m-Mentha-1(7),8-diene	0,14
30,0723	3-(p-hydroxy-m-methoxyphenyl)-2-propenal	0,06
30,1739	(E)-2,3-DIMETHYL-4-(2',6',6'-TRIMETHYL-1',2'-EPOXYCYCLOHEX-1'-YL)-BUTA-1,3-DIENE	0,10
44,1328	p-Allylanisole	0,11

ANNEXURE:6 **The results for Tea Tree Organic oil Sample**

The concentration of the compounds is reported in terms of area percent (%)		
		Tea Tree Organic oil
Retention time (min)	**Library identification**	**Area %**
7,6948	alpha-Thujene	1,09
7,892	alpha-Pinene	2,51
9,4326	beta-pipene	1,06
10,1888	MYRCENE	0,87
10,511	alpha-phellandrene	0,57
11,0421	alpha-terpipene	10,83
11,269	PARA CYMENE	1,52
11,3904	BETA-PHELLANDRENE	1,82
11,4764	1,8-CINEOLE (Eucalyptol)	3,22
12,589	gamma-Terpinene	19,12
12,7731	CIS-SABINENE HYDRATE	0,04
13,4517	Terpinolene	3,96
13,7676	cis-sabinene hydrate	0,09
13,9119	Linalool	0,06
14,4838	p-Menth-2-en-1-ol	0,36
15,1636	1-TERPINEOL	0,18
16,4741	TERPINEOL-4	36,39

16,7296	BETA.-FENCHYL ALCOHOL	3,40
16,8202	cis-Piperitol	0,08
17,161	Piperitol isomer I	0,13
20,7216	Bicycloelemene	0,06
21,0571	alpha-Copaene	0,08
21,6503	(-)-ISOLEDENE	0,10
21,7241	alpha-Cubebene	0,17
21,799	Aristolene	0,12
22,1675	beta-elemene	0,03
22,5932	(-)-.ALPHA.-GURJUNENE	0,57
22,8272	.BETA.-CARYOPHYLLENE	0,53
23,0459	VALENCENE	0,09
23,1998	Selina-3,7(11)-diene	0,10
23,3242	(+)-AROMADENDRENE	1,49
23,4321	ALLOAROMADENDRENE	0,19
23,6153	delta-Cadinene	0,23
23,6766	ALPHA-HUMULENE	0,15
23,8649	AROMADENDRENE	0,73
24,2087	Ethanone, 1-[2,2-dimethyl-1-(3-methyl-1,3-butadienyl)bicyclo[2.1.0]pent-5-yl]-, (E)-	0,60
24,4926	.BETA.-SELINENE	0,12
24,5443	ALLOAROMADENDRENE	0,13
24,6425	.delta.-selinene	0,30
24,7627	BICYCLOGERMACRENE	2,35
24,8707	alpha-cadinene	0,20

25,4462	.DELTA.-CADINENE	2,08
25,6237	1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)-Naphthalene	0,27
26,2377	EPIGLOBULOL	0,10
26,4117	AROMADENDRENE VI	0,12
26,6602	(+) spathulenol	0,08
26,809	Viridiflorol	0,43
26,9834	gamma-Gurjunene	0,20
27,0421	3-Methylacetanilide	0,18
27,2319	1-ethylideneoctahydro-7a-methyl-, cis-1H-Indene	0,15
27,7024	3,4,5,6,7,8-hexahydro-7-methyl-1(2H)-Naphthalenone	0,18
27,8297	1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)-Naphthalene	0,26