COMBINED ANTIBACTERIAL AND ANTIFUNGAL ACTIVITIES OF *Eucalyptus citriodora* AND *Syzygium aromaticum* ESSENTIAL OILS

JACKSON KIPNGETICH KIRUI

SM 14/2291/08

A thesis submitted to Graduate School in Partial Fulfilment of the Requirements for the Award of Master of Science Degree in Biochemistry of Egerton University

NOVEMBER 2014

Egerton University

DECLARATION AND RECOMMENDATION

Declaration

This thesis is my original work and has not been presented before for an award of a degree in any institution.

Signed..... Date.....

Jackson Kipngetich Kirui (SM14/2291/08)

Recommendation

This thesis has been prepared under our supervision and has our approval to be presented for examination as per Egerton University regulations.

Signed..... Date.....

Prof. Raphael Ngure

Department of Biochemistry and Molecular Biology

Egerton University

Signed..... Date.....

Dr. Christine Bii

Centre for Microbiology Research

Kenya Medical Research Institute

COPYRIGHT

DEDICATION

To Judy, Sharon, Aaron and Ken for prayerfully standing with me throughout the difficult times occasionally experienced in the course of the study.

ACKNOWLEDGEMENT

I wish to sincerely thank Prof. Raphael Ngure for tirelessly guiding me from the initial and conceptual stages of this research to the ultimate conclusion of the study. I also wish to thank Dr. Christine Bii for allowing the use of Kenya Medical Research Institute (KEMRI) laboratories for data collection. Gratitudes also go to Dr. Charles Mutai from Masinde Muliro University for his constant encouragement and moral support. May I also acknowledge Dr. Beatrice Amugune of University of Nairobi (UON), Miss Evageline Mathiu and Mr. Keneth Mutai for their tremendous contributions during data collection and analysis. I equally wish to thank the Director, Kenya Medical Training College (KMTC) for partial sponsorship of the study. More importantly I wish to thank the Almighty God for walking me through the turbulent journey and lifting me up every time the weight of incessant struggle appeared too much to bear.

ABSTRACT

The emergence of multi-drug resistant strains is a formidable threat to the fight against skin diseases and hence effective alternative regimes must be sought. Although many studies have been done on antimicrobial activities of *Syzygium aromaticum* and *Eucalyptus citriodora* oils, no information is available on their antimicrobial interaction and hence the purpose of this study. Bioactivity testing of *Syzygium aromaticum* and *Eucalyptus citriodora* oils was done using disc diffusion technique while the minimum inhibitory concentrations (MIC), the minimum bactericidal concentration (MBC) and the minimum fungicidal concentration (MFC) were determined by broth micro-dilution technique. Synergistic activity of the combination was determined using the Chequerboard assay. MRSA (IZD; 23 mm) and *E. coli* (IZD; 20 mm) were found to be extremely sensitive to *Eucalyptus citriodora* oil. *Staphylococcus aureus* ATCC 25923 (IZD; 19 mm), E. *coli* (IZD; 18 mm) and MRSA (IZD; 16 mm) were found to be very sensitive to *Syzygium aromaticum* oil. However *Pseudomonas aeruginosa* (IZD; 8 mm) was resistant to both *Eucalyptus citriodora* and *Syzygium aromaticum* oils. The fungi *Microsporum gypseum* (IZD; 20 mm) was extremely sensitive to *Eucalyptus citriodora* oil and this activity was more than for nystatin standard. *Candida albicans* ATCC 90028 (IZD; 15 mm) was very sensitive to *Syzygium aromaticum* oil. *Eucalyptus citriodora* exhibited the lowest MIC of 3.125% v/v against *Staphylococcus aureus* ATCC 25923 whlie *Syzygium aromaticum* oil exerted activity at the lowest MIC (1.56% v/v) against *Staphylococcus aureus* ATCC 25923, *E. coli* and *Candida albicans* ATCC. *Eucalyptus citriodora* oil had bactericidal activity against *Staphylococcus aureus* ATCC 25923 and MRSA and bacteristatic effect against *E. coli*. The oil had fungicidal effect against *Candida albicans* ATCC 90028. *Syzygium aromaticum* oil exhibited bactericidal activity against *Staphylococcus aureus* ATCC 25923 and bacteristatic effect on *E. coli*. It demonstrated fungicidal effect on *Microsporum gypseum* and *Trichophyton mentagrophytes* but had fungistatic activity against *Candida albicans* ATCC 90028 and *Cryptococcus neoformans*. The combination of the oils exhibited synergistic activity against MRSA (FICI; 0.48), *Staphylococcus aureus* ATCC 25923 (FICI; 0.48), *Microsporum gypseum* (FICI; 0.36) and *E. coli* ATCC 25922 (FICI; 0.50) but demonstrated indifferent activity against *Candida albicans* ATCC 90028 (FICI; 2.04). This study demonstrates that *Eucalyptus citriodora* and *Syzygium aromaticum* oils possess synergistic activity and may be combined for enhanced antimicrobial activity against skin diseases such as candidiasis, impetigo, acne and boils.

TABLE OF CONTENTS

DECLARATION AND RECOMMENDATION……..ii
COPYRIGHT.....iii
DEDICATION.....iv
ACKNOWLEDMENT.....v
ABSTRACT.....vi
TABLE OF CONTENTS.....vii
LIST OF TABLES.....x
LIST OF PLATES.....xi
LIST OF ABBREVIATIONS.....xii
CHAPTER ONE.....1
INTRODUCTION.....1
1.1 Background of Information.....1
1.2 Statement of the Problem.....4
1.3 General Objective.....5
1.3.1 Specific objectives.....5
1.4 Hypotheses.....5
1.5 Justification.....6
CHAPTER TWO.....7
LITERATURE REVIEW.....7
2.1 Antimicrobials.....7
2.2 Advantages of Medicinal Plant Products.....7
2.3 Antimicrobial Activities of *Eucalyptus citriodora* and *Syzygium aromaticum* Essential oils.....8
2.4 Mode of Action of Essential Oil Constituents……....9
2.5 Mode of Action of Cloramphenicol and Nystatin……..14
2.6 Skin Infections……14
2.7 Synergism in Antimicrobial Compunds……14
CHAPTER THREE.....15
MATERIALS AND METHODS……..11
3.1 Essential Oils.....15
3.2 Test Organisms.....15
3.3 Antibacterial *In-vitro* Assay.....15
3.4 Antifungal *In-vitro* Assay.....16

3.5 Minimum Inhibitory Concentrations of the Oils.....16
3.6 Combined Antibacterial Activity of the Oils……..16
3.7 Minimum Bactericidal and Fungicidal Activity.....17
3.8 Statistical Analysis.....17
CHAPTER FOUR.....18
RESULTS.....18
4.1 *In-vitro* Inhibitory Activity of *Syzygium aromaticum* and *Eucalyptus citriodora* Oils against the Test Bacteria.....18
4.2 *In-vitro* Inhibitory Activity of *Syzygium aromaticum* and *Eucalyptus citriodora* Oils Against the Test Fungi.....20
4.3 The Minimum Inhibitory Concentrations of *Syzygium aromaticum* Oil Against the Test Microbes.....21
4.4 The Minimum Inhibitory Concentrations of *Eucalyptus citriodora* Oil Against the Test Microbes.....22
4.5 Minimum Bactericidal/ Fungicidal Activity of *Syzygium aromaticum* Oil.....23
4.6 Minimum Bactericidal/ Fungicidal Activity of *Eucalyptus citriodora* Oil.....24
4.7 *In-vitro* Inhibitory Activity of a Combination of *Syzygium aromaticum* and *Eucalyptus citriodora* Oils at Various Concentrations Against Both the Test Bacteria and Fungi.....24
4.8 Synergistic Antimicrobial Activity between *Syzygium aromaticum* and *Eucalyptus citriodora* Oils.....25
CHAPTER FIVE.....27
5.0 DISCUSSION.....27
5.1 *In-vitro* Inhibitory Activity of *Syzygium aromaticum* and *Eucalyptus citriodora* essential Oils against the Test Bacteria and Fungi……..30
5.2 Minimum Inhibitory concentrations of *Syzygium aromaticum and Eucalyptus citriodora* Essential Oils Against the Test Bacteria and Fungi……..30
5.3 Minimum Bactericidal and Minimum Fungicidal Activity of

Eucalyptus Citriodora and *Syzygium aromaticum* Oils…… ….30
5.4 Combined Antimicrobial and Synergistic Activity of *Eucalyptus Citriodora* with *Syzygium aromaticum* Oil…….....32
CHAPTER SIX.....33
6.0 CONCLUSSIONS.....33
6.1 RECOMMENDATIONS.....35
REFERENCES.....36
APPENDICES.....48

LIST OF TABLES

Table 1: Shows mean zones of inhibition (mm) of *Syzygium aromaticum* and *Eucalyptus citriodora* oils at neat concentrations against the test bacteria……..18

Table 2: Shows Mean Zones of Inhibition (mm) of *Syzygium aromaticum* and *Eucalyptus citriodora* oils at neat concentrations against the test fungi……...20

Table 3: Shows minimum inhibitory concentrations of *Syzygium aromaticum* against both bacteria and fungi……..……...21

Table 4: Shows minimum inhibitory concentrations of *Eucalyptus citriodora* oil against both bacteria and fungi…….22

Table 5: Showing minimum bactericidal concentrations and minimum fungicidal concentrations of *Syzygium aromaticum* oil against the test microbes……..23

Table 6: Showing minimum bactericidal concentrations and minimum fungicidal concentrations of *Eucalyptus citriodora* oil against the test microbes……...24

Table 7: Shows the minimum inhibitory concentrations of a combination of *Eucalyptus citriodora* and Syzygium aromaticum oils at various concentrations against the test microorganism…….25

Table 8: Shows the Chequerboard determination of the combined antimicrobial activity of *Eucalyptus citriodora* and *Syzygium aromaticum* Oils…….26

LIST OF PLATES

Plate 1: A photograph of *Eucalyptus citriodora* plant……...3

Plate 2: A photograph of *Syzygium aromaticum* plant……...4

Plate 3: Shows zones of inhibition produced by *Eucalyptus citriodora* oil (clockwise direction) against *Staphylococcus aureus* ATCC 25923……..19

Plate 4: Shows zones of inhibition produced by *Syzygium aromaticum* oil (clockwise direction) against *Esherichia coli* ATCC 25922……...19

Plate 5: Showing MIC of *Syzygium aromaticum* oil against *Staphylococcus aureus* ATCC 25923.....22

LIST OF ABBREVIATIONS

ATCC	American type culture collection
CLSI	Clinical and Laboratory Standards Institute
DMSO	Dimethyl sulfoxide
FIC	Fractional inhibitory concentration
FICI	Fractional inhibitory concentration index
IZD	Inhibition zone diameter
KAVI	Kenya acquired immune deficiency syndrome vaccine initiative
KEMRI	Kenya Medical Research Institute
MBC	Minimum bactericidal concentration
MFC	Minimum fungicidal concentration
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*

CHAPTER ONE
INTRODUCTION

1.1 Background Information

Dermatological disorders are common in many countries (Komba and Mgonda, 2010) and are among the most frequent causes of morbidity (Accorsi *et al*, 2009). A variety of skin infections are seen in general practice (Sippe, 1991) and the types of organisms that cause primary skin and soft tissue infections include bacterial, viral and fungal pathogens as well as parasites (Laube, 2004). Acute bacterial skin infections are common (Gabillot and Roujeau, 2007) and most of them are usually caused by *Staphylococcus aureus* and *Streptococcus pyogenes* (Sharma and Verma, 2001). Bacterial skin infections commonly encountered in the community include impetigo, folliculitis/furunculosis, simple abscesses and other non-necrotizing cellulitis (Bernard, 2008).

Skin disorders are an important problem in children living in developing countries (Marrone *et al*, 2012) and those with disabilities may be particularly susceptible (Fathy *et al*, 2004). Methicillin-resistant *Staphylococcus aureus* has emerged as a cause of infection among otherwise healthy children and adults in the community where skin and soft tissue infections are most common (Gorwitz, 2008). Elderly individuals have an increased susceptibility to skin infections due to age-related anatomical, physiological and environmental factors (Laube, 2004) which include thinning, decreased secretions and reduced immune function (Weissmann, 1989).

Nursing home-acquired skin and soft tissue infections are common, with an estimated prevalence of approximately 5% (Lertzman and Gaspari, 1996). New treatments for skin disease continue to evolve (Thiers, 1998) but the identification of appropriate and novel antimicrobials is continually challenged by the emergence of antimicrobial resistance among bacteria, fungi and parasites (Yang and Kerdel, 2006).

The increase in microorganisms that have developed resistance to currently available antimicrobial agents has become a major cause of concern worldwide (Finch and Hunter, 2006) and especially in Africa (Aristide *et al*, 2011). Antimicrobial resistance continues to increase and many diseases are becoming increasingly difficult to treat as medicines become less effective resulting in a steady depletion of otherwise potent drugs that are currently available (Abdel-Hameed, 2003).

Bacterial infections are responsible for 90% of infections found in health care services (Lacmata *et al*, 2012) and the emergence of multi-drug resistant phenotypes is a major public health problem today in the treatment of bacterial infections (Fankam *et al*,

2011). Bacteria resistant to almost all of the available antibacterial agents have been identified (Bax *et al*, 2000) and serious skin and soft tissue infections caused by multidrug resistant pathogens have become more common in recent years (Raghavan and Linden, 2004). Indeed, the emergence of multidrug-resistant isolates in tuberculosis, acute respiratory infections, diarrhea coupled with the presence of a high percentage of hospital-acquired infections caused by highly resistant bacteria such as methicillin-resistant *Staphylococcus aureus* are becoming uncontrollable and could derail the progress made towards achieving the Millennium Development Goals set for 2015 (Dickson *et al*, 2011). In addition multidrug resistant *Staphylococcus aureus* strains now pose serious problems to hospitalized patients and their care providers (Fernandes *et al*, 2012). Emerging resistance to antibiotics such as norfloxacin, ciprofloxacin and amoxicillin-clavulanic acid by *Pseudomonas aeruginosa* and the increasing resistance among *Candida* species and other yeasts to available antifungal agents have also been reported (Moshi *et al*, 2007). Some of the greatest global health achievements like treating tuberculosis, human immunodeficiency virus (HIV)/AIDS (acquired immuno deficiency syndrome), pneumonia, diarrhoea and other infectious diseases are equally at risk as drug resistance rises thereby threatening the management of these infections (Norrby *et al*, 2005). Resistance of these micro-organisms to antimicrobial therapies reduces the effectiveness of the drugs leading to increased morbidity, mortality and health care expenditure (Richard and Joana, 2002).

The indiscriminate and widespread antimicrobial use continues to cause significant increase in drug-resistant and multidrug-resistant bacteria (Okoye *et al*, 2012) and clinicians have noted an association between antimicrobial resistance and antimicrobial use since the introduction of these agents over 50 years ago (Powers, 2003). The evolutionary response of bacteria, fungi, viruses and parasites to the selective pressure exerted by antimicrobial agents is the emergence of populations that resist the action of the antimicrobial (Sheldon, 2003).

Micro-organisms may develop resistance by changing the permeability of the cell wall so that the antibiotic does not get absorbed by the cell or it may develop certain types of enzymes which could destroy the antibiotic (Arnold, 2009).

Another mechanism of drug resistance involves expressing higher levels of target protein through increased transcription and translation or gene amplification. Resistance could also occur through increased drug efflux (Johnstone and Longley, 2005) since some micro-organisms may pump out the absorbed drug or suppress drug activation mechanisms (Ouellette, 2001).

The continuous escalation of resistant bacteria against a wide range of antibiotics has generated a considerable interest in the search for novel therapeutic compounds from medicinal plants (Njume *et al*, 2011). The search for drugs has accelerated in recent years (Marjorie, 1999) and plants are amongst promising resources for finding new antibacterial agents (Aliahmadi *et al*, 2011) whose use as natural antimicrobial agents is gaining popularity (Fullerton *et al*, 2011). These natural products may give new antimicrobial agents with possibly novel mechanisms of action with the potential of addressing multiple targets with low level of resistance in addition to being cost–effective (Navan and Shukla, 2011) since therapy with synthetic antibiotics is normally associated with high cost (Aristide *et al*, 2011). Conventional antibiotics have certain undesirable side effects including gastric ulceration and fat redistribution coupled with many pathogens adapting evasive mechanisms against a number of compounds over time (Dickson *et al*, 2011). Many plant-derived essential oils are known to exhibit antimicrobial activity against a wide range of bacteria and fungi (Sean *et al*, 2001). They include oils from the *Eucalyptus citriodora* and clove plants.

Plate 1: *Eucalyptus citriodora* tree

Eucalyptus oil has a history of wide application including pharmaceutical use. It has been shown to have antibacterial effect on pathogenic micro-organisms (Salari *et al*, 2006). The oil

has equally shown improved skin antiseptic properties when combined with chlorhexidine digluconate (Lambert *et al*, 2008). The clove tree from *Syzygium aromaticum* is also from the *Myrtaceae* family and has also been known for its analgesic, local anaesthetic, anti-inflammatory and antibacterial effects (Hemaiswarya and Doble, 2009).

Plate 2: *Syzygium aromaticum* tree

The significant antimicrobial properties of both *Syzygium aromaticum* and *Eucalyptus citriodora* essential oils make them ideal candidates for this study.

1.2 Statement of the Problem

Staphylococcus aureus is the leading cause of skin infection while *Pseudomonas aeruginosa* is the most common opportunistic bacteria that infects the skin and equally the most difficult to treat. Equally, *Candida albicans* is the major cause of superficial mycoses and can be troublesome in patients with wounds and underlying diseases such as diabetes. Other notably problematic pathogens include skin-invading molds which belong mainly to the genera *Microsporum* and *Trichophyton.* The presence of antibiotic-resistant *Staphylococcus aureus*, the emerging resistance to broad spectrum antibiotics by *Pseudomonas aeruginosa* and the increasing resistance among *Candida* species and other yeasts to available antifungal drugs present a health care challenge in terms of health-care cost and hospital stay. Such multi-drug resistant strains therefore pose serious challenges to the health care systems particularly in resource constrained third world countries. It is

therefore necessary to search for new and effective treatment options. In recent years *Eucalyptus citriodora and Syzygium aromaticum* oils have been investigated and shown to have antimicrobial properties and could therefore present cheap and effective therapeutic options for skin diseases when used in combination.

1.3 General Objective

To determine possible synergistic antimicrobial activity of *Eucalyptus citriodora* and *Syzygium aromaticum* oils against selected skin pathogens.

1.3.1 Specific objectives

1. To determine the *in-vitro* antibacterial and antifungal activities of *Eucalyptus citriodora* and *Syzygium aromaticum* essential oils, alone and in combination.
2. To determine the minimum inhibitory concentrations (MIC) of *Eucalyptus citriodora* and *Syzygium aromaticum* essential oils, alone and in combination.
3. To establish the minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of *Eucalyptus citriodora* and *Syzygium aromaticum* essential oils.
4. To determine the synergistic activity between *Eucalyptus citriodora* and *Syzygium aromaticum* essential oils.

1.4 Hypotheses

1. *Eucalyptus citriodora* and *Syzygium aromaticum* essential oils do not have antibacterial and antifungal activity alone and in combination.
2. The MIC of *Syzygium aromaticum* and *Eucalyptus citriodora* oil is not different.
3. The MBC and MIC of *Syzygium aromaticum* and *Eucalyptus citriodora* oil is not different.
4. *Eucalyptus citriodora* and *Syzygium aromaticum* essential oils do not have any synergistic activity.

1.5 Justification

Skin diseases account for over 10% of all diseases seen by family physicians. Despite the high frequency, skin disorders have so far not been regarded as a significant health problem in the development of public health strategies although morbidity is significant resulting in reduction in the quality of life. There is also concern over the rising prevalence of pathogenic microorganisms becoming resistant to conventional antimicrobials. Alternative treatment regimes are therefore required. Natural plant products have shown significant antimicrobial potentials and hence could provide therapeutic options. In addition, a large number of plant extracts have exhibited synergism. Although *Syzygium aromaticum* and *Eucalyptus*

citriodora oils as single entities have demonstrated potency against some of the test micro-organisms, no synergistic activity between the two oils has been evaluated and hence the aim of this study.

CHAPTER TWO
LITERATURE REVIEW

2.1 Antimicrobials

Antibiotics are one of the most important weapons in fighting bacterial infections (Nayan and Shukla, 2011) but the successive introduction of various antibiotics into therapeutic use has seen the sensitivity of pathogenic microorganisms changing (Aristide *et al*, 2011). The excessive (Trombetta *et al*, 2005) and indiscriminate antimicrobial use continues to cause significant increase in drug-resistant and multidrug-resistant bacteria (Okoye *et al*, 2012). This emergence of multidrug–resistant bacteria poses major health problems (Tiago *et al*, 2012) and has created a situation in which there are few or no alternative treatment options (Pankaj *et al*, 2011). This therefore necessitates constant development of newer agents, which can treat resistant organisms (Trivedi *et al*, 2004).

The use of and search for drugs derived from plants have accelerated in recent years (Cowan, 1999) and is gaining popularity (Fullerton *et al*, 2011). Plants have been used for many centuries for the treatment of ailments (Akiyemi *et al*, 2006) and knowledge of their chemical constituents is desirable (Ayoola *et al*, 2008) since they produce a wide variety of compounds which may have antimicrobial properties (Javad and Atefeh, 2010). Medicinal plants are sources of phytochemicals which are able to initiate different biological activities (Konate *et al*, 2012) thereby inhibiting bacterial growth by mechanisms different from presently-used treatment regimens (Njume, 2011) thereby representing an unparalleled reservoir of molecular diversity to drug discovery and development (Dickson *et al*, 2011).

2.2 Advantages of Medicinal Plant Products

Presently in the developing countries, synthetic drugs are not only expensive and inadequate for the treatment of diseases (Osanaiye *et al*, 2007) but suffer from various drawbacks in terms of toxicity, drug-drug interactions, lack of efficacy, cost and emergence of resistant strains as a result of frequent use (Pinto *et al*, 2009). The use of plant products has many advantages such as low cost of production, being biodegradable, easily obtained and less toxic (Musa *et al*, 2007). In recent years, medicinal plants have represented a primary health source for the pharmaceutical industry. A significant proportion of pharmaceutical products in current use are derived from plants (Mbah *et al*, 2006) and no less than 400 compounds are currently used in the preparation of drugs such as Vincristine and Vinblastine (Ajose, 2007). Phytomedicines derived from plants have shown great promise in the treatment of intractable infectious diseases including viral infections (Al-Bayati, 2008).

2.3 Antimicrobial Activities of *Eucalyptus* and *Syzygium aromaticum* Essential Oils

The essential oils and various extracts of plants have been of great interest for their potential uses as alternative remedies for the treatment of many infectious diseases (Javad and Batooli, 2010). Essential oils have been long recognized for their antibacterial, antifungal, antiviral, insecticidal and antioxidant properties (Juliani and Bassol, 2012). In recent years, a large number of essential oils and their constituents have been investigated for their antimicrobial properties against some bacteria and fungi (Kalemba and Kunicka, 2003).

The genus *Eucalyptus* is a source of several unique secondary metabolites, which show a variety of biological activities, such as those of antioxidants, antibacterials and HIV inhibitors (Javad and Atefeh 2010). The essential oil and its major component 1, 8-cineole has been found to have antimicrobial effects against many bacteria, viruses and fungi. It has been shown to act against gram negative and gram positive bacteria including *Esherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Trivedi and Hotchandani, 2004). The oil exhibits moderate to high activity against *Staphylococcus aureus*, *Escherichia coli*), *Candida albicans* but shows no activity against *Pseudomonas aeuruginosa* (Javad and Batooli, 2010). The oil has also been demonstrated to be active towards *Cryptococcus neoformans*, *Candida albicans* and *Trychophyton rubrum* (Lugman *et al*, 2008). It has also been shown to have activity against MRSA (Sporer *et al*, 2011).

Eucalyptus oil has also demonstrated efficacy against hospital-acquired isolates and reference strains particularly *Staphylococcus*, *Streptococcus* and *Candida* strains (Rasooli *et al*, 2009). *Eucalyptus maculate* and *Eucalyptus viminalis* have been established as being active against *Staphylococcus aureus* including MRSA, *Bacillus cereus*, *Propionibacterium acnes* and *Trichophyton mentagrophytes* therefore effective against micro-organisms that cause acne and athlete's foot (Takahashi *et al*, 2004). According to Tohidpour *et al* (2010) the essential oils and monoterpenes of *Eucalyptus globulus* were found to be effective against clinical isolates of methicillin-resistant *Staphylococcus aureus* suggesting an additional option to treat MRSA infections. *Eucalyptus tereticornis* has been identified as being a source of monoterpenoid rich oil exhibiting anti-oxidant activity (Sing *et al*, 2009) and in addition to their medicinal activities could also serve as a natural mosquitocide (Senthil, 2007) while *Eucalyptus sideroxylon* and *Eucalyptus torquata* have antimicrobial and antitumor properties (Ashour, 2008).

Clove oil is an essential oil from the clove plant, *Syzygium aromaticum*. The oil has also been shown to have considerable efficacy against *Staphylococcus*, *Streptococcus* and *Candida* strains (Springer *et al*, 2009). Clove oil has been shown to exhibit considerable effects against *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Dorman and Deans, 2005). Clove oil has equally exhibited significant antifungal activity against *Microsporum canis, Trichophyton rubrum, Epidermophyton floccosum* and *Microsporum gypseum* (Park *et al*, 2007). The oil and eugenol have shown considerable antifungal activity against other clinically relevant fungi, including fluconazole-resistant strains and thus deserving further investigation for clinical application in the treatment of fungal infections (Pinto *et al*, 2009). The oil has exhibit activity against *Escherichia coli, Staphylococcus aureu* but showed no activity against *Pseudomonas aeruginosa* (Gupta *et al*, 2008). However, Bari *et al* (2008) demonstrated activity of the oil against *Pseudomonas euruginosa* in addition to *Escherichia coli* and *Staphylococcus aureus*. *Syzygium aromaticum* has equally been shown to possess marked germicidal effect against *Candida albicans, Escherichia coli, Staphylococcus aureus* (Briozzo *et al*, 1989) and unlike in the study by Gupta *et al*, (2008) *Pseudomona aeruginosa* demonstrated sensitivity. Interestingly, Kamal and Radhika (2010) found the oil to be inactive against *Staphylococcus aureus* and *Candida albicans*. Ayoola *et al* (2008) and Sudsadu *et al* (2010) equally demonstrated activity of *Syzygium aromaticum* oil against *Staphylococcus aureus, Escherichia coli* and *Candida albicans*.

2.4 Mode of Action of Essential oil Constituents

Plants produce a variety of compounds with antimicrobial activity. Some are always present while others are produced in response to microbial invasion or physical injury (Roller, 2003). Identifying the most active antimicrobial compounds of essential oils is cumbersome because essential oils are complex mixtures of many different constituents (Delaquis *et al*, 2002; Djenane *et al*, 2011; Espina *et al*, 2011), and the composition of a particular essential oil may vary depending on the season of harvest, and the methods used to extract the oil. (Nannapaneni *et al*, 2009; Pereira and Meireles, 2010; Sánchez *et al*, 2010; Demuner *et al*, 2011; Djenane *et al*, 2011; Paibon *et al*, 2011).

Most studies concerning the antimicrobial mode of action of essential oil constituents have been performed on bacteria, while less is known about their action on yeast and molds. Gram-negative bacteria are generally less susceptible than Gram-positive bacteria (Trombetta *et al*, 2005). The outer membrane of Gram-negative bacteria contain hydrophilic

lipopolysaccharides (LPS), which create a barrier toward macromolecules and hydrophobic compounds, providing Gram-negative bacteria with higher tolerance toward hydrophobic antimicrobial compounds like those found in essential oils (Nikaido, 2003). Most essential oil constituents have several targets and it is therefore difficult to predict how susceptible a microorganism is and why the susceptibility varies from strain to strain (Hyldgaard *et al*, 2012). However, the following is known about the mode of action of some selected essential oil constituents.

Terpenes

Terpenes are hydrocarbons produced from combination of several isoprene units (C5H8) that are synthesized in the cytoplasm of plant cells and include p-cymene, limonene, terpinene, sabinene, and pinene (Hyldgaard *et al*, 2012). Terpenes do not represent a group of constituents with high inherent antimicrobial activity and they are inefficient as antimicrobials when applied as single compounds. (Bagamboula *et al*, 2004).

p-Cymene

The carvacrol precursor p-cymene is a monoterpene that has a benzene ring without any functional groups on its side chains and is not an efficient antimicrobial compound when used alone (Mann *et al*, 2000; Aligiannis *et al*, 2001; Bagamboula *et al*, 2004), but it potentiates the activity of compounds like carvacrol (Ultee *et a*l, 2002; Rattanachaikunsopon and Phumkhachorn, 2010) and polymyxin B nonapeptide (Mann *et al*, 2000). Several studies indicate that p-cymene is likely to act as a substitutional impurity in the membrane, which partly perturbs the membrane of microorganisms (Ultee *et al*, 2002. Investigations on cell and vesicle systems confirm that p-cymene has no effect on the membrane permeability, but cause a decrease in the enthalpy and melting temperature of membranes (Cristani *et al*, 2007), supporting the hypothesis that p-cymene acts as a substitutional impurity in the membrane.

Even though the action of p-cymene on the cell membrane is well established it has been shown to have a negligible effect on the protein synthesis of *E. coli* cells (Burt *et al*, 2007), while its effect on the membrane potential resulted in decreased cell motility, as a proton motive force is needed for flagellar movement (Gabel and Berg, 2003; Burt *et al*, 2007).

Terpenoids

Terpenoids are terpenes that undergo biochemical modifications via enzymes that add oxygen molecules and move or remove methyl groups and include thymol, carvacrol, linalool, linalyl acetate, citronellal, piperitone, menthol, and geraniol (Caballero *et al*, 2003). The antimicrobial activity of most terpenoids is linked to their functional groups, and it has been shown that the hydroxyl group of phenolic terpenoids and the presence of delocalized electrons are important for antimicrobial activity (Dorman and Deans, 2000; Ultee *et al*, 2002; Ben Arfa *et al*, 2006). Exchanging the hydroxyl group of carvacrol with methyl ether affects its hydrophobicity, antimicrobial activity, and changes how the molecule interacts with the membrane (Veldhuizen *et al*, 2006). Carvacrol's antimicrobial activity is comparable to that of 2-amino-p-cymene, which indicates that the hydroxyl group is important, but not essential for carvacrol's activity (Veldhuizen *et al*, 2006). The antimicrobial activity of essential oils can often be correlated to its content of phenolic constituents (Aligiannis *et al*, 2001; Kalemba and Kunicka, 2003; Rhayour *et al*, 2003).

The terpenoids are a large group of antimicrobial compounds that are active against a broad spectrum of microorganisms, with the most active monoterpenoids identified so far being carvacrol and thymol (Dorman and Deans, 2000).

Thymol

Studies have shown that thymol interacts with cell membranes thus affecting membrane permeability and this has been documented by loss of membrane potential, cellular uptake of ethidium bromide, and leakage of potassium ions, ATP, and carboxyfluorescein (Lambert *et al*, 2001; Walsh *et al*, 2003; Xu *et al*, 2008). Although the protective properties of lipopolysaccharide (LPS) against thymol had been confirmed using random transposon-insertion mutants, treatment of *E. coli* cells with thymol caused release of LPS and disruption of the outer membrane (Shapira and Mimran, 2007). Thymol integrates at the polar head-group region of a lipid bilayer causing alterations to the cell membrane, which at low concentrations induce adaptational changes in the membrane lipid profile in order to compensate for thymol's fluidifying effects and to maintain the membrane function and structure (Turina *et al*, 2006; Di Pasqua *et al*, 2007).

The mode of action of thymol against yeast and fungi is through disruption of vesicles and cell membranes and impaired ergosterol biosynthesis which consequently affects cell membrane integrity because ergosterol regulates membrane fluidity and asymmetry similarly to cholesterol in animal cells (Cristani *et al*, 2007; Ahmad *et al*, 2011).

Interestingly, thymol has been demonstrated to induce cell lysis and to only alter the cell structure of proliferating S. cerevisiae cells, indicating the effect of thymol depends on cell proliferation (Bennis *et al*, 2004). Contrary to this, Rao *et al* (2010) proposed that thymol activates specific signaling pathways in yeast by causing cytosolic Ca2+ bursts and transcription responses similar to Ca2+ stress and nutrient starvation (Rao *et al*, 2010).

Carvacrol

Carvacrol has been demonstrated to affect the outer membrane of Gram-negative bacteria (La Storia *et al*, 2011). Disintegration of the outer membrane caused release of LPS from Gram-negative bacteria (Helander *et al*, 1998). Although carvacrol affects the outer membrane, its site of action is thought to be the cytoplasmic membrane, resulting in passive transport of H+ into the cell cytoplasm and transporting K+ back out (Ultee *et al*, 2002; Ben Arfa *et al*, 2006).

Besides the interaction with membranes, carvacrol has been proposed to interact with membrane proteins and periplasmic enzymes (Juven *et al*, 1994).

Phenylpropenes

The phenylpropenes that have been most thoroughly studied are eugenol, isoeugenol, vanillin, safrole, and cinnamaldehyde and comparison of molecules that are chemically similar to eugenol and isoeugenol indicated that the free hydroxyl groups are important for their activity against bacteria, but not yeast (Laekeman *et a*l, 1990). Some of isoeugenol's activity might be attributed to the double bond in the α and β positions of the side chain, and a methyl group in the γ position (Jung and Fahey, 1983). Furthermore, the antimicrobial activity of phenylpropenes depends on the kind and number of substituents on the aromatic ring, selected microbial strains, and the experimental test parameters such as choice of growth medium, temperature, etc. (Pauli and Kubeczka, 2010).

Eugenol

Eugenol is a major constituent in clove essential oil, and its antimicrobial activity is linked to its ability to permeabilize the cell membrane and interact with proteins (Walsh *et al*, 2003; Gill and Holley, 2006a; Hemaiswarya and Doble, 2009). As recently demonstated eugenol induced minor changes in the fatty acid profile of *Pseudomonas fluorescens*, *E. coli*, *Brochotrix thermosphacta*, *S. enterica*, and *S. aureus*, and cell damages to *E. coli* and *B. thermosphacta* cells (Di Pasqua *et al*, 2007). Eugenol has also proven to inhibit the activity

of ATPase, histidine decarboxylase, amylase, and protease (Gill and Holley, 2006b). Inhibition of the ATPase may be important for cell killing at high eugenol concentrations because energy generation needed for cell recovery is impaired (Gill and Holley, 2006b). Eugenol is known to depend on cell proliferation and has been shown to alter cell membrane and cell wall structures of proliferating S. cerevisiae cells resulting in the release of cellular content (Bennis *et al*, 2004).

Cinnamaldehyde

Aldehyde groups are reactive and have the ability to cross-link covalently with DNA and proteins through amine groups, thereby interfering with their normal function (Feron *et al*, 1991). It has been established that cinnamaldehyde binds to the FtsZ protein, inhibiting its GTP dependent polymerization and thereby preventing cell division (Domadia *et al*, 2007; Hemaiswarya *et al*, 2011). Cinnamaldehyde is also thought to interact with cell membrane and has been demonstrated to alter the membrane lipid profile with large increases in saturated fatty acids and yielding a more rigid membrane and cell structure of *E. coli*, *S. enterica*, *P. fluorescens*, and *B. thermosphacta*, while only *S. aureus* demonstrated disintegration of the cell envelope (Di Pasqua et al, 2007). Among fungi, the primary mode of action of cinnamaldehyde has also been proposed to be inhibition of cell division (*Bang et al*, 2000).

Vanillin

Vanillin has been shown to inhibit respiration *of E. coli* and Listeria innocua cells, and to disrupt the potassium and pH homeostasis of *Lactobacillus plantarum* cells (Fitzgerald *et al*, 2004). Propidium iodide staining demonstrated that treatment with vanillin disrupted membrane integrity of only a sub-population of cells and it was proposed that although vanillin primarily is a membrane active compound, it may also have intracellular target sites (Fitzgerald *et al*, 2005). Not much is known about vanillin's mechanism of antifungal activity, but it has been suggested that the aldehyde moiety of vanillin plays an important role in its antifungal activity as seen in *S. cerevisiae* which converted vanillin into vanillic acid and vanillyl alcohol which possessed no antimicrobial activity thereby confirming the key-role of the aldehyde moiety (Feron *et al*, 1991; Fitzgerald *et al*, 2005).

2.5 Mode of Action of Chloramphenicol and Nystatin

Chloramphenicol is thought to interfere competitively with the binding of the aminoacyl-tRNA 3′-terminus to ribosomal A-site (Maria et *al*, 2003) leading to inhibition of mitochondrial translation (Sheth *et al*, 2014).

Nystatin is a membrane-active polyene macrolide antibiotic that induces membrane permeability by forming complexes with ergosterol located in fungal membranes, leading to intracellular leakage and cell death (Yamasaki *et al*, 2011).

2.6 Skin Infections

Skin infections are common (Moulin *et al*, 2008) and include bacterial skin infections which are commonly encountered in most fields of medicine (Tan *et al*, 1998) particularly in pediatrics with various presentations and severity (Gabillot-Carré and Roujeau , 2007). Skin and soft tissue infections are usually caused by *Staphylococcus aureus* and *Streptococcus pyogenes* (Sharma and Verma , 2001) and infections due to *Staphylococcus aureus* have recently become a public concern due to emerging resistance against widely used antibiotics (Wiese-Posselt *et al*, 2007). Community-associated methicillin-resistant *Staphylococcus aureus* is increasing in incidence (Demos *et al*, 2012) and is a rapidly spreading pathogen associated predominantly with skin infections (Vingsbo Lundberg and Frimodt-Møller, 2013) among otherwise healthy children and adults in the community (Gorwitz, 2008).Bacterial skin infections commonly encountered in the community include impetigo, folliculitis/furunculosis, simple abscesses, erysipelas and other nonnecrotizing cellulites (Stulberg, 2002; Bernard, 2008).

Impetigo is a common, superficial bacterial skin infection seen in general practice (George and Rubin 2003) and is most frequently encountered in children (Koning *et al*, 2012) and characterised by an inflamed and infected epidermis (Sladden and Johnston, 2005). Impetigo is caused by *Streptococcus* or *Staphylococcus* and can lead to lifting of the stratum corneum resulting in the commonly seen bullous effect (Stulberg *et al*, 2002). In mild and localized impetigo topical antibiotics are recommended whereas in widespread or severe one and in ecthyma systemic antibiotics like, cloxacillin, erythromycin, azithromycin or cephalexin should be used (Sharma and Verma , 2001).

Cellulitis is an acute bacterial non-necrotizing dermal-hypodermal infection predominantly affecting the lower limbs (Blum and Menzinger, 2013) and is a common condition seen by physicians (Gunderson, 2011). Cellulitis is an infection of the dermis and

subcutaneous tissue that has poorly demarcated borders and is usually caused by *Streptococcus* or *Staphylococcus* species (Stulberg *et al*, 2002). Burn wound cellulitis is the second leading complication reported in burns (Heard *et al*, 2014). cellulitis is an acute infections of dermal and subcutaneous tissues caused most frequently by Group A beta-hemolytic *streptococci* (erysipelas) or *S. aureus* requiring systemic antibiotics like oral or intravenous penicillin, erythromycin, cephalexin, cloxacillin, vacomycin, minocycline or ciprofloxacin depending upon the severity, suspected causative organism and culture/sensitivity results (Sharma and Verma , 2001).

Dermatophytoses are considered to be one of the major public health problems in the world (Naseri *et al*, 2013) and are the most common of the superficial fungal infections (Surendran *et al*, 2014). Superficial fungal infections due to dermatophytes are common over the world (Budak *et al*, 2013) and the continuously changing epidemiology of invasive fungal infections results in the need for an expanded armamentarium of antifungal therapies (Abdul , 2012). Although dermatophytoses can appear at any age, some types are particularly prevalent in children (Triviño-Duran, 2005).

The incidence of topical fungal infections has progressively increased in recent years primarily because of an increased number of immunocompromised patients and the increased use of health clubs and community swimming pools, which favour the spread of infections (Raghu *et al*, 2014). Pathogens responsible for skin mycoses are primarily anthropophilic and zoophilic dermatophytes from the genera *Trichophyton* , *Microsporum* and *Epidermophyton* (Havlickova *et al*, 2008).

Tinea cruris and *tinea corporis* are common fungal infections seen by both general practitioners and dermatologists (van Zuuren *et al*, 2014). *Tinea cruris*, a pruritic superficial fungal infection of the groin, is the second most common clinical presentation for dermatophytosis and can be treated with a variety of topical antifungals (Jones *et al*, 2014)

Tinea capitis is the most common dermatophyte infection of the scalp affecting mainly children and rarely adults (Ginter-Hanselmayer *et al*, 2007) whose causative agents vary within different geographical areas (Binder *et al*, 2011) and is an increasing problem in Europe (Hackett *et al* 2006).

Pityriasis versicolor is a common superficial fungal infection of the skin caused by *Malassezia* species (Lyakhovitsky *et al*, 2013) which are yeast that naturally colonizes on the skins surface (Gupta and Lyons, 2014). Although *pityriasis versicolor* is the only human disease for which *Malassezia* yeasts have been fully established as pathogens, it is still not clear which species are implicated (Crespo-Erchiga *et al*, 2008). Despite the fact that a range

of molecular methods have been developed as tools for the diagnosis of *Malassezia* species, there are several drawbacks associated with them, such as inefficiency of differentiating all the species, high cost, and questionable reproducibility (Vuran *et al*, 2014). The diagnosis is made clinically by its classic appearance of round or oval macules with fine scale that may be hyperpigmented or hypopigmented (Cantrell and Elewksi, 2014).

A wide range of topical antifungal drugs are used to treat these superficial dermatomycoses(El-gohary *et al*, 2014) but recurrence following successful treatment remain high and there are no dosage guidelines available for administration of systemic antifungal agents that carry risks of adverse events (Gupta and Lyons 2014).

2.7 Synergism in Antimicrobial Compounds

Synergy occurs when a blend of two antimicrobial compounds has an antimicrobial activity that is greater than the sum of the individual components (Morten *et al*, 2012). Combination antimicrobial therapy is needed and employed to broaden the spectrum of coverage (Moellering, 1983; (Suliman *et al*., 2009. The concept of combination therapy may be advantageous when resistance to single agent develops rapidly (Eliopoulos and Moellering, 1982). One approach developed to overcome this mechanism of resistance consists of combining an efficient but possibly unstable antibiotic with a powerful inhibitor of the inactivating enzyme (Labia *et al*, 1990). Exploiting synergies between several compounds has been suggested as a solution to this problem (Hyldgaard *et al*, 2012). For instance, fosfomycin in combination with linezolid and vancomycin has shown synergistic activity against *Staphylococcus aureus* (Fernandez *et al*, 2011). Tea extracts have equally demonstrated synergistic activity with chloramphenicol, gentamycin, methicillin and nalidixic acid against *Shigella dysenteriae, Yersina enterocolitica* and *Escherichia coli* (Tiwari *et al*, 2005). However, the extract weakened the antibacterial effect of amoxicillin in methicillin-resistant *Staphylococcus aureus* infected mice (Yao, 2010).

The combinations of *Alphinia galangal* with either *Rosemarynus officinalis* or *Eucalyptus staigerana* extracts has exhibited synergistic activity and could be used as natural antimicrobials (Dykes *et al*, 2011). Various synergistic antimicrobial activities have been reported for constituents or fractions of essential oils (Delaquis *et al*, 2002). It has also been shown that clove and rosemary essential oils have synergistic effects against *Staphylococcus epidermidis* and *Candida albicans* (Sun *et al*, 2007). The combination of *Thymus vulgaris* and *Pimpinella anisum* essential oils has shown additive action against *Pseudomonas aeruginosa* (Al-Bayati, 2007) however, no synergistic or antagonistic antimicrobial activity

has been documented between clove and *Eucalyptus* oils although they have potential antimicrobial properties with no reported toxicological side effects making them ideal candidates for this study. It is envisaged that any synergism between the oils will mitigate against the problem of drug resistance.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Essential Oils

Eucalyptus citriodora (eucalyptus oil) and *Syzygium aromaticum* (clove oil) essential oils were used in this study. The oils were obtained as steam distillates from Bell sons and Co. Druggists Ltd (Southport, England)

3.2 Test Organisms

Four bacterial strains consisting of *Staphylococcus aureus* ATCC 25923, methicillin resistant *Staphylococcus aureus* **(**clinical isolate), *Esherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* were used in the study. The fungal strains used included reference and clinical isolates of *Candida albicans* (ATCC 90028), and dermatophytes from clinical sources namely *Microsporum gypseum*, *Trichophyton mentagrophytes* and *Cryptococcus neoformans*. The fungal strains were culture collections at Mycology Laboratory, Centre for Microbiology Research, Kenya Medical Research Institute (KEMRI).

3.3 Antibacterial *In-vitro* Assay

In-vitro antimicrobial activities of clove and *Eucalyptus* oils, alone and in combination were performed using the disc diffusion method, while the minimum inhibition concentration (MIC) was determined by broth micro-dilution technique (Bauer, 1996; CLSI, 2003). The procedure for the *in vitro* assay was as described by CLSI (2003). Briefly, from a 24-hour culture of the test bacteria, 0.5 McFarland standard suspensions were prepared. The suspension was spread uniformly using a sterile wire loop on to a sterile Muller Hinton agar plate so as to achieve a confluent growth. Sterile 6mm paper discs impregnated with 20 µl of essential oil were then aseptically placed on the surface of the inoculated medium. The plates were then incubated at 4 ^{0}C for 1 hour to allow for diffusion of the oil into the medium and then at 37 ^{0}C for another 24 hours to enable the micro-organisms to grow and interact with the oil. The inhibition zone diameters (IZD) were measured and recorded at the end of the incubation time. The tests were done in triplicate and chloramphenicol (30µg) was used as the positive control (Cook, 2008; Tatjana *et al*, 2010; Aljeboury, 2013) while 5 % dimethyl sulphoxide diluent was used as the negative control.

3.4 Antifungal Quantitative *In-vitro* Assay

In-vitro antifungal activity of *Syzygium aromaticum* and *Eucalyptus citriodora* essential oils, singly and in combination was performed using disc diffusion assay and the MIC was determined using broth micro-dilution method (Bauer, 1996, CLSI 2003). The procedure for the *in vitro* assay was as described by CLSI (2003). Briefly, from a 24 hour culture of yeasts and 72 hour culture of dermatophytes, 0.5 McFarland standard suspensions were prepared and spread uniformly on Sabourauds dextrose agar. A sterile 6mm paper discs impregnated with 20 µl of the test oil was aseptically placed on the surface of the inoculated medium. Plates were incubated at 4 ^{0}C for 1 hour to allow diffusion of oil and then at 35 ^{0}C for 24 hours for yeast and at 30 ^{0}C for 72 hours for molds. After incubation, the plates were then examined for zones of inhibition. The IZD around each disc was measured and recorded at the end of incubation time. The tests were carried out in triplicate and nystatin (23µg) was used as positive antifungal standard (Cook, 2008; Tatjana *et al*, 2010) while 5% DMSO was used as negative control. All microbiological procedures were performed at the opportunistic infection laboratory at Centre for Microbiology Research. The procedures were done under level 2 containment facility and under KEMRI biosafety guidelines.

3.5 Minimum Inhibitory Concentration

To determine MIC, the oils were serially diluted in a 96 microtitre well using 5% DMSO as the diluent to give concentration ranges of 0.78-50 % (v/v). Sterile discs impregnated with 20 µl of the various concentrations were aseptically placed on the surface of the medium which had been inoculated with standard suspensions of the microorganisms and incubated as outlined in sections 3.3 for bacteria and 3.4 for fungi. The lowest concentration without growth after incubation was taken as the MIC.

3.6 Combined Antibacterial Activity

To assess for any synergism, the oils were mixed at various concentrations: 100:0% (v/v), 80:20% (v/v), 60:40% (v/v), 50:50% (v/v), 40:60% (v/v), 20:80% (v/v), and 0:100% (v/v) of *Eucalyptus citriodora* and *Syzygium aromaticum* oils respectively and antibacterial and antifungal activity of each concentration determined using disc diffusion method as outlined in section 3.3 and 3.4. Chequerboard assay (Henry *et al*, 2009) was used to assess for synergistic activity of *Eucalyptus citriodora* in combination with *Syzygium aromaticum*.

3.7 Minimum Bactericidal and Fungicidal Activity of *Eucalyptus citriodora* and *Syzygium aromaticum* Oils

To determine the minimum bactericidal (MBC) or minimum fungicidal concentrations (MFC), the oils were serially diluted in a 96 microtitre well as outlined in section 3.5 above after which 100µl of bacterial or fungal standard suspension was added into each serial dilution. To each microwell, 100µl of Muller Hinton agar (for MBC) or Sabouraud's dextrose agar (MFC) was added. The mixture was incubated at respective conditions as outlined in section 3.3 and 3.4 for bacteria and fungi respectively. At the end of the incubation period, the mixture from each microwell was picked using a sterile wire loop and applied on the surface of antibiotic-free media consisting of Muller Hinton for bacteria and Sabouraud's dextrose agar for fungi. The plates were then incubated at the respective temperature and time. At the end of the incubation period the lowest concentration that prevented growth was taken as the MBC or MFC.

3.8 Statistical Analysis

The means and the standard deviations of the inhibition zone diameters of *Syzygium aromaticum* and *Eucalyptus citriodora* oils when acting alone and in combination against the tested micro-organisms were computed and used to compare their antimicrobial activities. Student's t -test test was used to ascertain if the p values from inhibition zones were statistically significant at 95% confidence level ($p<0.05$). The minimum inhibitory concentrations (MIC) were evaluated and used to compare the effectiveness of the oils against the test pathogens. Chequerboard assay was performed and Fractional inhibitory concentration index (FICI) computed and used to determine exhibition of any synergistic activity between the two oils on the test microorganisms.

CHAPTER FOUR
RESULTS

4.1 *In-vitro* Inhibitory Activity of *Syzygium aromaticum* and *Eucalyptus citriodora* Oils Against the Test Bacteria.

Both *Syzygium aromatium* and *Eucalyptus citriodora* essential oils were found to be effective against nearly all the test bacteria (Table 1). Based on the inhibition zone diameter (IZD) values, the microorganisms were placed into four categories: microorganisms having IZD ≤ 8 mm, considered resistant; microorganisms with IZD between 9-14 mm, considered sensitive; microorganisms with IZD between 15-19 mm, considered very sensitive; while microorganisms with IZD ≥ 20 mm were considered extremely sensitive to the oil (Babu *et al*, 2011)).

Table 1: Shows mean zones of inhibition (mm) of *Syzygium aromaticum* and *Eucalyptus citriodora* oils at neat concentrations against the test bacteria.

S.No.	**Microorganism**	***Syzygium aromaticum***	***Eucalyptus citriodora***	**Chloramphenicol (30 µg)**
1	*Staphylococcus aureus*	*18±2.2	*19±1.9	25
2	*Escherichia coli*	*18±2.0	*20±1.9	25
3	MRSA	**16±2.1	**23±1.9	28
4	*Pseudomonas aeruginosa*	*8±1.9	*8±2.0	19

Chloramphenicol was used as positive control and DMSO (5%) as the negative control. Each value is the average of three independent replicates.*****Antimicrobial activity not significantly different at p≤0.05. **Antimicrobial activity significantly different at p≤0.05.

From the results MRSA (IZD; 23 mm) and *E. coli* (IZD; 20 mm) were found to be extremely sensitive to *Eucalyptus citriodora* oil while *Pseudomonas aeruginosa* (IZD; 8 mm) was resistant. *Staphylococcus aureus* ATCC 25923 (IZD; 19 mm) was very sensitive to

Eucalyptus citriodora oil. *E. coli* (Plate 4: IZD; 18 mm), *Staphylococcus aureus* ATCC 25923 (IZD; 18 mm) and MRSA (IZD; 16 mm) were found to be very sensitive to *Syzygium aromatic* oil and like *Eucalyptus citriodora* oil *Pseudomonas aeruginosa* (IZD; 8 mm) was resistant to *Syzygium aromatic* oil. MRSA (IZD; 28 mm), *E. coli* (IZD; 25 mm) and *Staphylococcus aureus* (IZD; 25 mm) were extremely sensitive to chloramphenicol while *Pseudomonas euruginosa* (IZD; 19 mm) was very sensitive to this reference drug.

There was a significant difference ($p \leq 0.05$) in activity between the oils against MRSA with *Eucalyptus citriodora* oil showing higher activity ($p=0.013$) than *Syzygium aromaticum* oil against the bacteria. However, there was no difference ($p \leq 0.05$) in activity between the oils against *Staphylococcus aureus* ($p=0.547$) and *E. coli* ($p=0.281$) (Appendix 2). Chloramphenicol showed higher activity against all the test bacteria than the two oils.

Plate 3: Shows zones of inhibition produced by *Eucalyptus citriodora* oil (clockwise direction) against *Staphylococcus aureus* ATCC 25923. Chloramphenicol was used as the positive control. *Eucalyptus* oil had an IZD of 19mm compared to 18mm for *Syzygium aromaticum* and 25mm for the positive control. EU: *Eucalyptus* oil, C-ve; Negative control, Sau; *Staphylococcus aureus,* C+ve; Positive control, 1, 2, 3; Triplicate zones of inhibition, MH; Muller Hinton agar.

Plate 4: Shows zones of inhibition produced by *Syzygium aromaticum* oil (clockwise direction) against *Esherichia coli* ATCC 25922. Chloramphenicol was used as the positive control. *Syzygium aromaticum* oil had an IZD of 18mm compared with 20mm for *Eucalyptus* and 25mm for the positive control.

4.2 *In-vitro* Inhibitory Activity of *Syzygium aromaticum* and *Eucalyptus citriodora* Oils Against the Test Fungi

Syzygium aromaticum and *Eucalyptus citriodora* oils exhibited significant activity against the test fungi (Table 2). *Microsporum gypseum* (IZD; 20mm) was extremely sensitive to the *Eucalyptus citriodora* oil and interestingly, the oil was more active than nystatin standard. However, *Cryptococcus neoformans* (IZD; 15mm) was less sensitive to *Eucalyptus citriodora* oil. *Candida albicans* ATCC 90028 (18 mm) and *Trichophyton mentagrophytes* (IZD; 18 mm) were very sensitive to *Eucalyptus citriodora* oil. *Candida albicans* ATCC 90028 (15 mm)was very sensitive to *Syzygium aromaticum* oil while *Cryptococcus neoformans* (IZD; 10mm) was less sensitive to the oil. *Microsporum gypseum* (IZD; 11mm) and *Trichophyton mentagrophytes* (IZD; 14mm) were moderately sensitive to *Syzygium aromaticum* oil. *Trichophyton mentagrophytes* (IZD; 27 mm), *Candida albicans* (IZD; 22mm) and *Cryptococcus neoformans* (IZD; 21mm) were extremely sensitive to nystatin while *Microsporum gypseum* (IZD; 19mm) was moderately sensitive.

Table 2: Shows mean zones of inhibition (mm) of *Syzygium aromaticum* and *Eucalyptus citriodora* oils at neat concentrations against the test fungi

.	**Test micro-organism**	*Syzygium*	*Eucalyptus*	Nystatin

Serial No.		*aromaticum*	*citriodora*	(23 µg)
1	*Microsporum gypseum*	**11±2.4	**20±2.0	19
2	*Trichophyton mentagrophytes*	*14±2.2	*18±1.8	27
3	*Candida albicans*	*15±2.0	*18±2.0	22
4	*Cryptococcus neoformans*	**10±0.4	**15±2.0	21

Nystatin was used as positive control and DMSO as the negative control. Each value is the average of three independent replicates. *****Antimicrobial activity not significantly different at $p \leq 0.05$. **Antimicrobial activity significantly different at $p \leq 0.05$.

There was a significant difference ($p \leq 0.05$) between the activity of the oils against *Microsporum gypseum* and *Cryptococcus neoformans* with *Eucalyptus citriodora* oil being more active against both *Microsporum gypseum* (p=0.008) and *Cryptococcus neoforman* (p=0.013). Nystatin showed higher activity than the oils against *Trichophyton mentagrophytes*, *Cryptococcus neoformans* and *Candida albicans*.

4.3 The Minimum Inhibitory Concentrations of *Syzygium aromaticum* Oil Against the Test Microbes

The MIC determination of *Syzygium aromaticum* was tested at different concentrations (Table 3). The minimum concentration required to prevent the growth of each microorganism varied. The *Syzygium aromaticum* oil exhibited the lowest MIC of 1.56% v/v against both *Staphylococcus aureus* ATCC 25923 and *E. coli* while acting at a higher MIC of 6.25% v/v against MRSA. For fingi, *Syzygium aromaticum* oil acted at the lowest MIC 0f 0.78% v/v against Candida *albicans* ATCC 90028 and the highest MIC against *Trychophyton mentagrophites* (12.5% v/v). The oil demonstrated moderate MIC of 3.125 % v/v and 6.25% v/v against *Cryptococcus neoformans* and *Microsporum gypseum* respectively.

Table 3: The minimum inhibitory concentrations of *Syzygium aromaticum* oil against both bacteria and fungi determined by zones of inhibition (mm).

	Serial dilutions of *Syzygium aromaticum* oil								
Micro-organism / inhibition zone diameter (mm)	**1:1**	**1:2**	**1:4**	**1:8**	**1:16**	**1:32**	**1:64**	**1.128**	**MIC (%v/v)**
S. aureus	24	23	19	19	18	15	0	0	1.56
E. coli	24	22	12	12	12	9	0	0	1.56
MRSA	19	18	14	14	0	0	0	0	6.25
M. gypseum	24	14	10	8	0	0	0	0	6.25
T. mentagrophytes	14	10	10	0	0	0	0	0	12.5
C. albicans	40	38	26	24	24	14	12	0	0.78
C. neoformans	34	30	28	16	12	0	0	0	3.125

NB: MIC not determined for *Pseudomonas aeruginosa*.

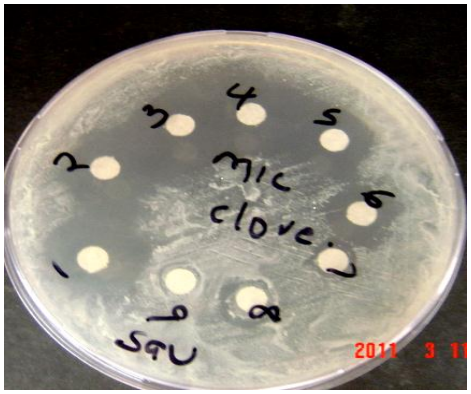


Plate 5: Showing MIC of *Syzygium aromaticum* oil on *Staphylococcus aureus* ATCC 25923. *****Sau; *Staphylococcus aureus*, 1-9: Zones of inhibition (clockwise direction).

4.4 The Minimum Inhibitory Concentrations of *Eucalyptus citriodora* Oil Against the Test Microbes

The MIC of *Eucalyptus citriodora* was tested at different concentrations (Table 4). The oil exhibited the lowest MIC (3.125% v/v) against *Staphylococcus aureus* while acting at a moderate MIC (6.25% v/v) against both *E. coli* and MRSA. The oil exhibited MIC of 12.5% v/v against both *Microsporum gypseum* and *Candida albicans*.

Table 4: The minimum inhibitory concentrations of *Eucalyptus citriodora* oil against both bacteria and fungi determined by zones of inhibition (mm).

Serial dilutions of *Eucalyptus citriodora* oil

Microorganisms /Inhibition Zone Diameter (mm)	**1:1**	**1:2**	**1:4**	**1:8**	**1:16**	**1:32**	**1:64**	**1:128**	MIC (%v/v)
S. aureus	19	17	16	12	9	0	0	0	**3.125**
E. coli	20	15	13	9	0	0	0	0	**6.25**
MRSA	23	22	20	10	0	0	0	0	**6.25**
M. gypseum	20	14	10	0	0	0	0	0	**12.5**

C. albicans	15	12	9	0	0	0	0	0	**12.5**

NB: MIC not determined for *Pseudomonas aeruginosa*, T*rychophyton mentagrophytes* and *Cryptococcus neoformans*.

4.5 Combined Mimimum Inhibitory Concentration of *Eucalyptus citriodora* with *Syzygium aromaticum* Oil

The MIC of *Eucalyptus citriodora* oil in combination with *Syzygium aromaticum* oil at ratios of 100:0, 80:20, 50:50, 60:40, 20:80 and 0:100 was determined against the test microorganisms. From the findings *Staphylococcus aureus* ATCC 25923 was found to be most sensitive to the combination with the lowest MIC of 0.25 % (v/v) followed by *E. coil* (MIC: 0.67% v/v) and MRSA (MIC: 1.5% v/v). For fungi *Mycrosporum gypseum* and *Candida albicans* each exhibited an MIC of 1.5% (v/v). The combination of the two oils exhibited activity against *Staphylococcus aureus*, *E. coil*, MRSA and *Mycrosporum gypseum* at lower MIC than the individual oils (Table 7).

Table 5: The minimum inhibitory concentrations of the combination of *Sysygium aromaticum* and *Eucalyptus citriodora* oils at various concentrations against the test microorganisms (%v/v)

Microorganism/ IZD (mm)	**100:0**	**80:20**	**50:50**	**60:40**	**40:60**	**20:80**	**0:100**	**MIC (%v/v)**
sa	24	19	14	11	10	9	0	**0.25**
Ec	21	16	11	10	9	0	0	**0.67**
MRSA	22	17	12	10	0	0	0	**1.5**
Mg	23	18	13	9	0	0	0	**1.5**
Ca	19	14	10	9	0	0	0	**1.5**

Key: *Cn-Cryptococcus neoformans*; *MRSA-Methicillin-resistant Staphylococcus aureus; Sa-Staphylococcus aureus; Ec- E. coli;Mg- Microphyton gypseum*.

IZD- Inhibition Zone Diameter

NB: Test not determined for *Pseudomonas aeruginosa*, *Candida albicans* and *Trichophyton mentagrophytes*.

4.6 Minimum Bactericidal and Minimum Fungicidal Activity of *Syzygium aromaticum* oil

In this study the minimum bactericidal and minimum fungicidal concentrations of *Syzygium aromaticum* oil were determined at various concentrations (Table 5)**.** At the concentration(s) where no growth occurred the oil was deemed to be bactericidal or fungicidal if the MBC/ MFC was the same or not more than fourfold higher than the MIC while it was considered to be bacteristatic or fungistatic at the concentration(s) where growth occurred and the MBC/ MFC was fourfold higher than MIC (Mathew 2004). From the findings *Syzygium aromaticum* oil exhibited bactericidal activity at a lower concentration of 1.56 % (v/v) against *Staphylococcus aureus* and bacteriostatic activity against *E. coli* at a concentration of 50% v/v.

For fungi the oil exhibited fungicidal activity at the lowest concentration of 6.25% (v/v) against *Microsporum gypseum* while being fungicidal at the highest concentration of 25% v/v against *Trychophyton mentagrophytes*. The oil exhibited fungistatic activity at concentrations of 12.5% and 25% v/v against *Candida albicans* ATCC 90028 and *Cryptococcus neoformans* respectively.

Table 6: Minimum bactericidal or fungicidal concentration(s) of *Syzygium aromaticum* oil against the test microorganism.

Serial dilutions of *Syzygium aromaticum* oil

Microorganisms /Dilutions	**1:1**	**1:2**	**1:4**	**1:8**	**1:16**	**1:32**	**1:64**	**1:128**	**MBC/MFC (%v/v)**
S. aureus	n/g	n/g	n/g	n/g	n/g	n/g	g/o	g/o	**1.56**
E. coli	n/g	g/o	g/o	g/o	g/o	g/o	g/o	g/o	**50**
MRSA	n/g	n/g	n/g	n/g	n/g	n/g	g/o	g/o	**1.56**
M. gypseum	n/g	n/g	n/g	n/g	g/o	g/o	g/o	g/o	**6.25**
C. albicans	n/g	n/g	n/g	g/o	g/o	g/o	g/o	g/o	**12.5**
C. neoformans	n/g	n/g	g/o	g/o	g/o	g/o	g/o	g/o	**25**
T. Mentagrophytes	n/g	n/g	g/o	g/o	g/o	g/o	g/o	g/o	**25**

n/g - no growth occured ; g/o - growth observed.

4.7 Minimum Bactericidal and Minimum Fungicidal Activity of *Eucalyptus citriodora* oil

The minimum bactericidal and minimum fungicidal concentrations of *Eucalyptus citriodora* oil was determined at various concentrations (Table 6)**.** From the findings the oil had bactericidal effect against *Staphylococcus aureus* ATCC 25923 and MRSA at concentrations of 6.25% v/v each. The oil was bacteriostatic against *E. coli* ATCC 25922 at a concentration of 50% v/v.

For fungi the oil exhibited bactericidal activity at a concentration of 25% v/v against Candida albicans ATCC 25923. at the lowest concentration against *Microsporum gypseum* (6.25% v/v) followed by *Cryptococcus neoformans* with a fungicidal concentration of 12.5 % (v/v) and Candida albicans at a concentration of 25 % (v/v) while exhibiting activity against *Trychophyton mentagrophytes* at the highest concentration of 50 % (v/v).

Table 7: Shows the minimum bactericidal and fungicidal concentrations of *Eucalyptus citriodora* oil against the test micro-organisms.

Serial dilutions of *Eucalyptus citriodora* oil

Microorganisms /Dilutions	**1:1**	**1:2**	**1:4**	**1:8**	**1:16**	**1:32**	**1:64**	**1:128**	**MBC/MFC (%v/v)**
S. aureus	n/g	n/g	n/g	n/g	g/o	g/o	g/o	g/o	**6.25**
E. coli	n/g	g/o	g/o	g/o	g/o	g/o	g/o	g/o	**50**
MRSA	n/g	n/g	n/g	n/g	g/o	g/o	g/o	g/o	**6.25**
M. gypseum	n/g	n/g	n/g	n/g	g/o	g/o	g/o	g/o	**6.25**
C. albicans	n/g	n/g	g/o	g/o	g/o	g/o	g/o	g/o	**25**
C. neoformans	n/g	n/g	n/g	g/o	g/o	g/o	g/o	g/o	**12.5**
T. Mentagrophytes	n/g	g/o	g/o	g/o	g/o	g/o	g/o	g/o	**50**

4.8 Synergistic Antimicrobial Activity Between *Eucalyptus citriodora* and *Syzygium aromaticum*

From the various concentrations (Table 7) the Chequerboard assay was used to assess for synergism between the two oils. For each microorganism, the *Eucalyptus citriodora*: *Syzygium aromaticum* oil combination was deemed synergistic, indifferent or antagonistic by calculating the Fractional inhibitory concentration (FIC) for each oil and then the FIC index (FICI) using the following formulae:

FIC= The MIC of antimicrobial in combination/ MIC of antimicrobial alone.

FICI= FIC of *Eucalyptus citriodora* + FIC of *Syzygium aromaticum oil*.

FICI≤ 0.5, synergistic; FICI> 0.5 or ≤ 4.0, indifferent; FICI> 4.0, antagonistic (Henry *et al*, 2009).

From the results synergistic activity was demonstrated with *Staphylococcus aureus* (FICI: 0.240, *E. coli* (FICI: 0.50), MRSA (FICI: 0.48), and *Microsporum gypseum* (FICI: 0.36) while the combination exhibited indifferent activity against *Candida albicans* (FICI: 2.04).

Table 8: Cheguerboard Determination of the Combined Antimicrobial Activity of *Eucalyptus citriodora* and *Syzygium aromaticum oils*

Mo/ Cn	**MIC of EC (%v/v) alone/in combination**		**FIC of EC**	**MIC of SA *oil*(%v/v) alone/ combination**		**FIC of SA**	**FICI**	**Result**
Sa								
EC+SA	3.125	0.25	0.08	1.56	0.25	0.16	0.24	Synergistic
Ec								
EC+SA	6.25	0.67	0.11	1.56	0.67	0.43	0.50	Synergistic
MRSA								
EC+SA	6.25	1.5	0.24	6.25	1.5	0.24	0.48	Synergistic
Pa								
EC+SA	Nd		Nd	Nd	Nd	Nd	Nd	Nd
Mg								

EC+SA	12.5	1.5	0.12	6.25	1.5	0.24	0.36	Synergistic
Ca								
EC+SA	12.5	1.5	0.12	0.78	1.5	1.92	2.04	Indifferent
Tm								
EC+SA	12.5	Nd	Nd	12.5	Nd	Nd	Nd	Nd
Cn								
EC+SA	3.125	Nd	Nd	3.125	Nd	Nd	Nd	Nd

Key: Nd- Not determined.; Mo- Microorganisms; EC- *Eucalyptus citriodora;*
FIC- Fractional inhibitory concentration; FICI- Fractional inhibitory concentration index
Cn- Combination of oils ; SA- *Syzygium aromaticum*

4.9 Correlation between the activity of *Syzygium aromaticum* Oil and its concentrations against the test microorganisns.

Log concentration-inhibition zone diameter and the absolute concentration-inhibition zone diameter curves for *Syzygium aromaticum* against the test microorganisms were plotted and regression analyses done to establish the relationship between the parameters. For all the microorganisms the R^2 values were above 0.7 suggesting that there is a linear relationship between the concentration of the oil and activity against the microorganisms.

Table 9: showing the inhibition zone diameters of *Syzygium aromaticum* at various concentrations against the test microorganisms

Concentration of oil (%v/v)	***Sau***	***E. coli***	**MRSA**	***Mg***	***Ca***	***Crypto***
	Inhibition Zone Diameters(mm)					
50	24	24	19	24	40	34
25	23	22	18	14	38	30
12.5	19	12	14	10	26	28
6.25	19	12	14	8	24	24
3.125	18	12	0	0	24	16
1.56	15	9	0	0	14	12

0.78	0	0	0	0	12	8

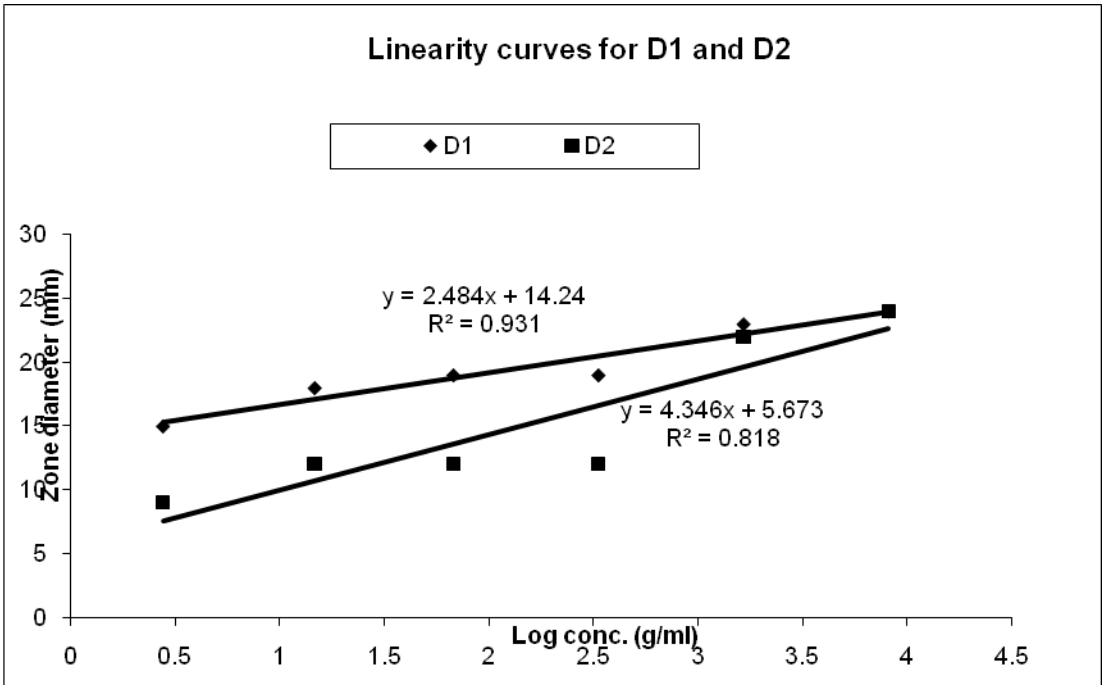


Fig 1: Showing Concentration-Inhibition Zone Diameter curves of *Syzygium aromaticum* against *Staphylococcus aureus* (D1) and *Escherichia coli* (D2).

Curve D1 has a linearity of y=2.484x + 14.24 and R^2 of 0.9318 with a linear range between 1.56 to 50 %(v/v).

Curve D2 has a linearity of y=4.3469x + 5.6735 and R^2 of 0.8184 with a linear range of between 1.56 to 50 %(v/v).

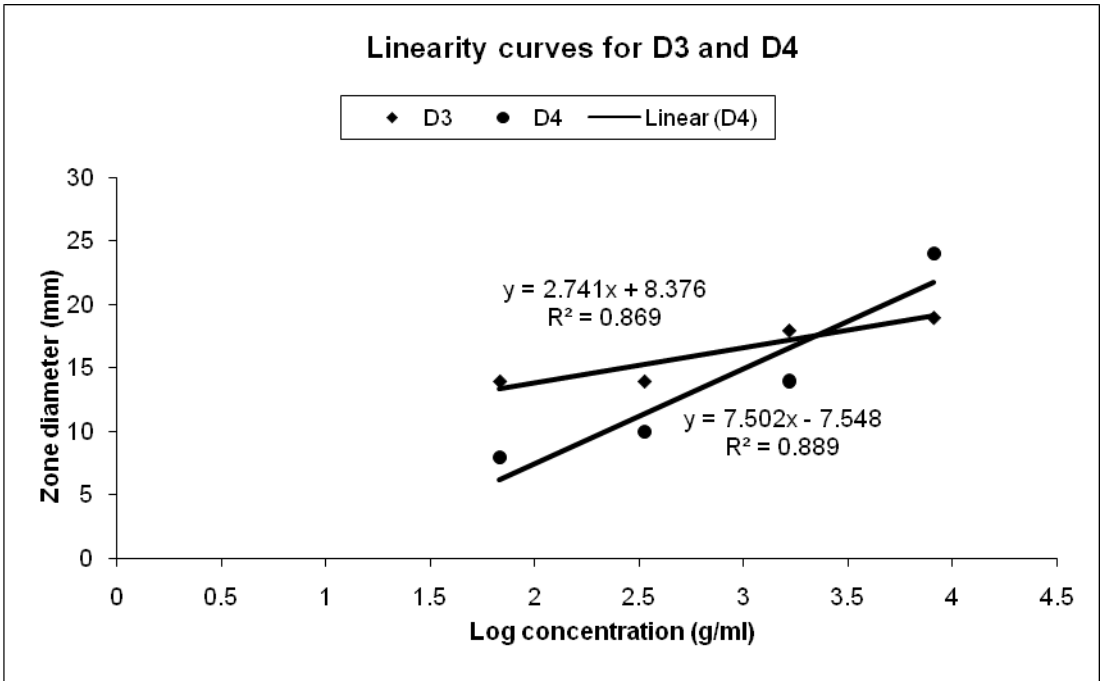


Fig 2: Showing Concentration-Inhibition Zone Diameter curves of *Syzygium aromaticum* against MRSA (D3) and *Microsporum gypseum* (D4).

Curve D3 has a linearity of y=2.741x + 8.3767 with R^2 of 0.8699 and a linear range of between 6.25 to 50g/ml. Curve D4 has a linearity of y=7.502x – 7.5481 with R^2 of 0.8895 and a linear range between 6.25 to 50 %(v/v).

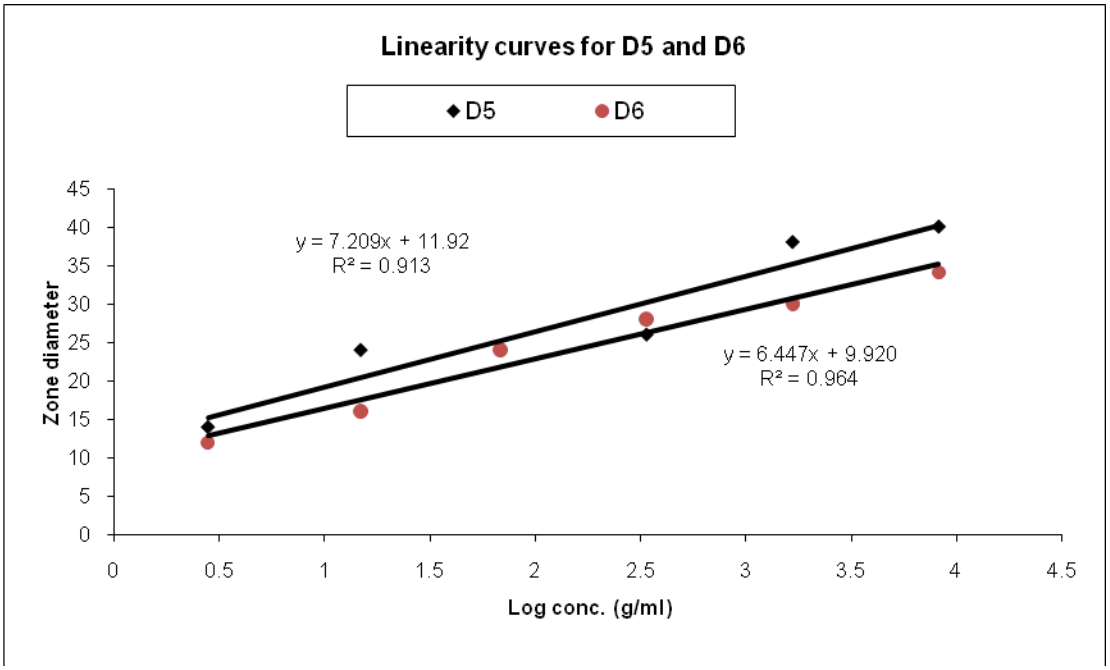


Fig 3: Showing Concentration-Inhibition Zone Diameter curves of *Syzygium aromaticum* oil against *Candida albicans* (D5) and *Cryptococcus neoformans* (D6).

Curve D5 has a linearity of y=7.2098x + 11.921 and R^2 of 0.9133 with a linear range between 0.7813 to 50g/ml. Curve D6 has a linearity of y=6.44x + 9.9205 and R^2 of 0.9643 with a linearity range between 0.7813 to 50g/ml.

Series 1 has a linearity of y=0.1603x+17.035 and R^2 of 0.796 with a linear range between 1.56 to 50g/ml. Series 2 has a linearity of y=0.311x+10.06 and R^2 of 0.86 with a linearity range between 1.56 to 50%(v/v).

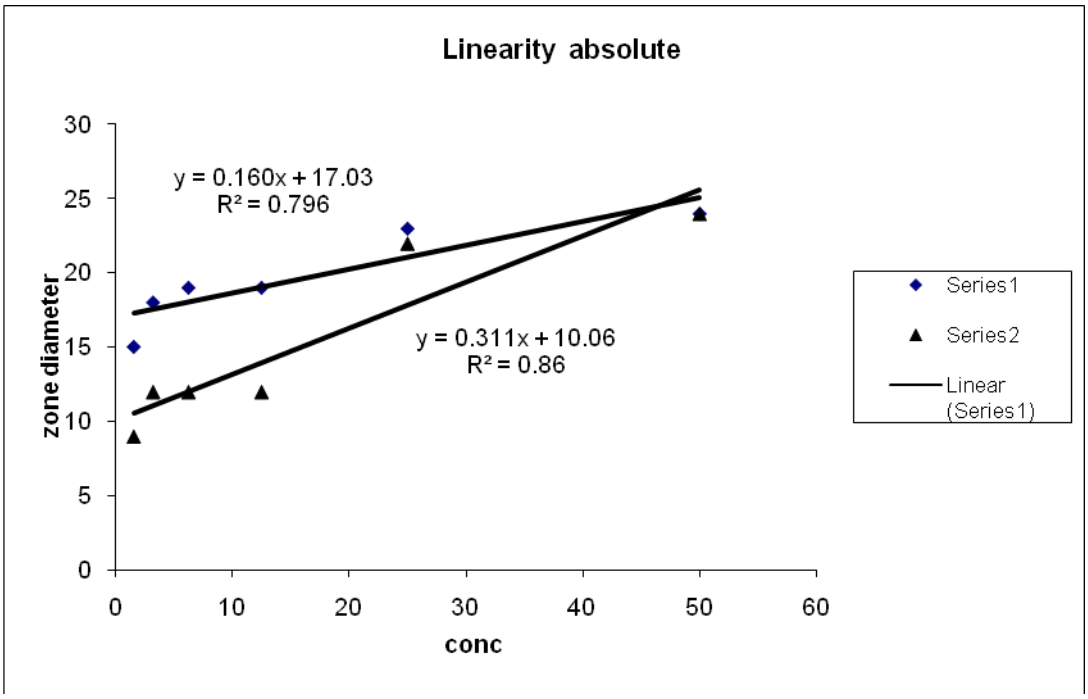


Fig 5:

Fig 4: Showing absolute linear Concentration-Inhibition Zone Diameter curves of *Syzygium aromaticum* oil against *Staphylococcus aureus* (series 1) and *Escherichia coli* (series 2).

Series 1 has a linearity of y=0.1603x+17.035 and R^2 of 0.796 with a linear range between 1.56 to 50g/ml. Series 2 has a linearity of y=0.311x+10.06 and R^2 of 0.86 with a linearity range between 1.56 to 50%(v/v).

CHAPTER FIVE

DISCUSSION

5.1 In-*vitro* Inhibitory Activity of *Syzygium aromaticum* and *Eucalyptus citriodora* Essential Oils Against the Test Bacteria and Fungi

The antimicrobial activity of plant oils and extracts has been recognized for many years (Hammer *et al*, 1999) and essential oils have been shown to possess antibacterial, antifungal, antiviral, insecticidal and antioxidant properties (Seenivasan *et al*, 2006). However no study has been done on synergistic antibacterial and antifungal activity between *Syzygium aromaticum* and *Eucalyptus citriodora* oils. In this study antibacterial and antifungal activity of the oils alone and in combination were tested. From the results, *Syzygium aromaticum* and *Eucalyptus citriodora* oils exhibited significant antimicrobial activity against *Staphylococcus aureus* ATCC 25923, MRSA, *Escherichia* coli ATCC 25922, *Candida albican* ATCC 90028, *Microsporum gypseum, Trichophyton mentagrophytes* and *Cryptococcus neoformans*. This concurs with earlier observations (Hammer *et al*, 1999; Sean *et al*, 2001) that many plant-derived oils exhibited antimicrobial activity against a wide range of bacteria and fungi. The high antimicrobial activity of the oils against the microorganisms may be explained by the presence of mixtures of plant metabolites with inherently broad antimicrobial activities. *Eucalyptus citriodora* oil contains terpenes which are known to be active against a wide variety of microorganisms, including gram-positive and gram-negative bacteria and fungi (Trombetta, 2005) and this may account for the broad spectrum of activity of *Eucalyptus citriodora* oil as documented in this study. Terpenes are known to exert their effects either by interfering with the integrity of cell memrane or through inhibition of protein sunthesis (Ultee *et al*, 2002; Burt *et al*, 2007) and this may partly account for the mode of action of *Eucalyptus citriodora* oil. Eugenol is a major constituent of Syzygium *aromaticum* oil with antimicrobial properties which occur through permeabilization of the cell membrane and interaction with proteins (Di Pasqua *et al*, 2007). This may account for the observed antimicrobial activity of Syzygium *aromaticum* oil.

Pseudomonas aeruginosa was, however, resistant to both *Syzygium aromaticum* and *Eucalyptus citriodora* oils and this observation is similar with findings of Gupta *et al* (2009) and Javad *et al* (2010). Indeed Moshi *et al* (2007) had similarly observed increasing resistance by *Pseudomonas aeruginosa* to norfloxacin, ciprofloxacin and amoxicillin-clavulanic acid. According to Abdel-Hameed (2003), this phenomenon of antimicrobial resistance continues

to increase and many diseases are becoming increasingly difficult to treat as medicines become less effective resulting in a steady depletion of otherwise potent drugs that are currently available. The resistance of *Pseudomonas aeruginosa* appears to be the result of an impermeable cell membrane and the presence of efflux mechanisms and porine-dependent inhibition that protect the bacteria against the action of the oils (Mayaudi *et al*, 2008). This could therefore explain for the failed activity of *Syzygium aromaticum* and *Eucalyptus citriodora* oils. More research work that could involve chemical structural modification could therefore be necessary in order to evade the targeting of the drug for elimination by the microorganism. However, *Pseudomonas aeruginosa* was found to be very sensitive to chloramphenicol and this could be as a result of a different metabolic pathway through which chloramphenicol exerts its effect.

The current study found the oils to be effective against MRSA which is supported by earlier observations made by Tohidpour *et al* (2010) and Sporer *et al* (2011). Indeed, MRSA was found to be extremely sensitive to *Eucalyptus citriodora* oil and as Trivedi *et al* (2004) noted, topical application of *Eucalyptus* oil proved effective against MRSA. This observation is particularly significant and suggests that *Eucalyptus citriodora* oil can be an alternative agent in the management of conditions associated with this highly resistant pathogen. MRSA was also found to be very sensitive to *Syzygium aromaticum* oil and is in agreement with Babu *et al* (2011). The continued sensitivity of MRSA to *Syzygium aromaticum* in addition to *Eucalyptus citriodora* may arise from known generally high susceptibility of gram-positive bacteria to antimicrobial agents and this could augment the fight against challenging infections caused by this rapidly evolving pathogen.

There was a significant difference in the activity between the oils against MRSA with *Eucalyptus citriodora* exhibiting better activity. This means that given a choice between the two oils in the management of suspected MRSA infection, *Eucalyptus citriodora* would do better. *Staphylococcus aureus* was equally very sensitive to both *Eucalyptus* and *Syzygium aromaticum* oils suggesting that the oils may be useful in treating infections such as boils and impetigo that are associated with the bacteria. This observation supports earlier findings of Javad and Batooli (2010), Babu *et al* (2011), Trivedi and Hotchandani (2004) and Kamal and Randhika (2010). Both oils were also found to be active against *E. coli* and this is in agreement with observations made by Trivedi and Hotchandani (2004), Javad and Batooli (2010), Gupta *et al* (2009) , Ayoola *et al* (2008) and Sudsadu *et al* (2010). *E. coli* was found to be extremely sensitive to *Eucalyptus citriodora* oil while Javad and Batooli (2010) observed the microbe as being very sensitive to the oil. The microbe was also very sensitive

to *Syzygium aromaticum* oil which is in agreement with Burt and Reinders (2003) and Gupta *et al* (2009). The sensitivity of *E. coli* to both oils is clinically significant as the bacterium is known to colonize wounds resulting in superinfection. There was, however, no significant difference in the activity between the two oils against S*taphylococcus aureus* ATCC 25923 and *E. coli* ATCC 25922 meaning that where one fails to provide therapeutic relief then the other oil may not be of any benefit.

Previous study (Eugénia, 2009) indicated that *Syzygium aromaticum and* eugenol have considerable antifungal activity against clinically relevant fungi, including fluconazole-resistant strains. In this study *Syzygium aromaticum* and *Eucalyptus citriodora* oils were found to be active against *Candida albican* ATCC 90028, *Microsporum gypseum, Trichophyton mentagrophytes* and *Cryptococcus neoformans*. Interesingly, *Eucalyptus citriodora* oil was found to be more active than both Syzygium *aromaticum* and the nystatin standard against *Microsporun gypseum*. The oil was also highly effective against *Trichophyton mentagrophytes*. This means that *Eucalyptus* oil can be used effectively to treat dermatophytoses such as *Tinea capitis, Tinea corporis* and *Tinea cruris*. Candida albicans was also found to be highly sensitive to *Eucalyptus citriodora* indicating that the oil can be used in the management of candidiasis and other opportunistic infections in patients who are immunocompromised for instance due to HIV/AIDS. The oil may also be beneficial in other cases involving suppression of the body immunity by broad spectrum antibiotics that may precipitate vaginal candidiasis. The sensitivity by the microorganisms to the oils supports similar observavations by Park *et al* (2007), Lugman *et al* (2008) and Pinto *et al* (2009). M*icrosporun gypseum* was equally very sensitive to nystatin. *Trichophyton mentagrophytes* was also found to be very sensitive to *Syzygium aromaticum* oil and this observation is similar to findings of Park *et al* (2007). *Candida albicans* ATCC 900028 was also found to be very sensitive to *Syzygium aromaticum* oil. These observations mean that *Syzygium aromaticum* can provide an alternative option in the management of dermatophytoses and candidiasis. The sensitivity of the micro-organism to both oils concurs with ealier observations by Ayoola *et al* (2008), Park *et al* (2007), Pinto *et al* (2009), Briozzo *et al* (1998), Javad and Batooli (2010), Lugman *et al* (2008) and Rasooli *et al* (2009). Both oils exhibited activity against *Cryptococcus neoformans* thus providing an alternative treatment for cryptococcosis which is a defining opportunistic infection in HIV/AIDS and is potentially fatal. Similar observations were made by Lugman *et al* (2008).

The fact that the two oils exhibited significant activity against the test microorganisms may suggest the usefulness of the oils in combating various skin diseases. More over, these oils

are cheap, readily available, easy to prepare and less toxic compared to conventional agents. In addition, there has been an increase in the number of microorganisms that have developed resistance to conventional drugs (Finch and Hunter, 2006).

5.2 Minimum Inhibitory Concentrations of *Syzygium aromaticum* and *Eucalyptus citriodora* Essential Oils Against the Test Bacteria and Fungi.

Syzygium aromaticum was most effective against *Staphylococcus aureus* ATCC 25923 and *E. coli* ATCC 25922 at MIC each of 1.56% (v/v) and moderately against MRSA (MIC: 6.25% (v/v). The activity against *Staphylococcus aureus* ATCC25923 by the oil was higher than that observed by Sudsadu *et al* (2010) of 2.5% v/v). This may be explained by variations in oil composition as this may be affected by the season of harvest or the methods used during extraction. The oil was also most effective against *Candida albicans* at MIC of 1.56% (v/v) compared with other fungi. *Eucalyptus citriodora* was also found to be most effective against *Staphylococcus aureus* ATCC25923 with MIC of 3.25 % (v/v) and least effective against *Microsporum gypseum* and *Candida albicans* ATCC 900028 each with MIC of 12.5% (v/v).However, when *Eucalyptus citriodora* and *Syzygium aromaticum* were combined at various concentrations (Table 7) the MIC of the combination was lower than for the individual oils against *Staphylococcus aureus* ATCC25923, E. coli ATCC 25922, MRSA and *Microsporum gypseum*. The fact that the two oils as single entities were effective at lower concentrations against Staphylococcus *aureus* ATCC25923 *E. coli* ATCC 25922 and at moderate concentrations against MRSA confirms that both oils are potential substitutes to conventional agents in the management conditions associated with the susceptible microorganisms. In addition their use may not be associated with side effects that are witnessed with large doses administered for common conventional therapeutic agents. However, better therapeutic results would be achieved when the oils are combined than when used as monotherapies. This would also minimise the development of resistance by the microorganisms as well as side effects as lower concentrations would be necessary to achieve the desired effect.

5.3 Minimum Bactericidal and Minimum Fungicidal Activity of *Eucalyptus citriodora* and *Syzygium aromaticum* oils.

In this study the minimum bactericidal and minimum fungicidal concentrations of *Syzygium aromaticum* oil were determined at various concentrations (Table 5)**.** At the concentration(s) where no growth occurred the oil was deemed to be bactericidal or fungicidal if the MBC/ MFC was

the same or not more than fourfold higher than the MIC while it was considered to be bacteristatic or fungistatic at the concentration(s) where growth occurred and the MBC/ MFC was fourfold higher than MIC (Mathew 2004). From the findings *Syzygium aromaticum* oil exhibited bactericidal activity at a lower concentration of 1.56 % (v/v) against *Staphylococcus aureus* and bacteriostatic activity against *E. coli* at a concentration of 50% v/v. It is noted that the MIC and MBC of *Syzygium aromaticum* oil against *Staphylococcus aureus* ATCC 25923 is equal (i.e. 1.56% v/v). This means that at such a low concentration the oil is able to kill the bacterium. This suggests that the oil can be the drug of choice in the management of infections caused by the bacterium such as boils, impetigo and cellulitis. For *E. coli* the oil was only able to suppress the growth of the bacterium at a very high concentration meaning that in infections where *E. coli* is suspected as the cause Syzygium *aromaticum* can not be the first- line drug.

For fungi the oil exhibited fungicidal activity at the lowest concentration of 6.25% (v/v) against *Microsporum gypseum* while being fungicidal at the highest concentration of 25% v/v against *Trychophyton mentagrophytes*. This means that the oil may not be very effective in *Trychophyton mentagrophytes* associated infections but can be recommended for treatment of Tinea capitis, Tinia coporis and other infections caused by *Microsporum gypseum*. The oil exhibited fungistatic activity at concentrations of 12.5% and 25% v/v against *Candida albicans* ATCC 90028 and *Cryptococcus neoformans* respectively. This indicates that the oil is effective at fairly low concentration against *Candida albicans* ATCC 90028 and can be useful in candidiasis particularly in HIV/AIDS patients. However, the oil may not be beneficial in managing Cryptococcosis in such patients because their immunity is already suppressed and yet the oil only suppresses the fungi.

For bacteria *Eucalyptus citriodora* oil exhibited bactericidal activity at lower concentrations (6.25 % v/v) against *Staphylococcus aureus* ATCC 25923 and MRSA. The fact that the MIC (3.25% v/v) and the MBC (6.25% v/v) of *Eucalyptus citriodora* oil against *Staphylococcus aureus* ATCC 25923 are significantly low implies that the oil is very effective against the microorganism and may be recommended for use in the management of diseases associated with the pathogen. For MRSA the MIC and MBC were equal (i.e. 6.25% v/v). This means the oil is able to kill the microorganism at fairly low concentration and this is a significant observation owing to the ease at which MRSA has developed resistance to a wide range of conventional drugs. For E. coli the oil could only suppress the growth of the microorganism at the same concentration as that of *Syzygium aromaticum* (50% v/v). It therefore implies that

Eucalyptus citriodora oil just like *Syzygium aromaticum* oil cannot be the drug of choice in E. coli associated infections.

For fungi the oil was fungicidal at concentration of 25% v/v against *Candida albicans* ATCC 90028 which implies that the oil may be useful in managing fungal skin diseases such as *Tinea cruris, Tinea capitis, Tinea versicolar* or *Tinea corporis* and other opportunistic fungal infections.

5.4 Synergistic Activity of *Eucalyptus citriodora* with Syzygium aromaticum oil.

Two drugs applied to a system can either give the same response as the sum of the two drugs individually (additive), a greater response (synergistic) or a smaller response (if one drug blocks the effects of the other) (Vijaya *et al*, 2007). In this study, the two oils were combined at different concentrations and their antibacterial and antifungal activity tested. The activities of the oils when acting alone and in combination were evaluated and the Chequerboard assay used to assess for synergism between the two oils. From the results synergistic activity was demonstrated with *Staphylococcus aureus* ATCC 25923 (FICI: 0.240), *E. coli* ATCC 25922 (FICI: 0.50) MRSA (FICI: 0.48), and *Microsporum gypseum* (FICI: 0.36). These observations suggest that the oils augment each other in their activity against these pathogens and hence better therapeutic response would be achieved with the combination as opposed to using the individual oils to treat conditions associated with the susceptible microorganisms. The combination would also ensure that the effective dosage is reduced and hence occurrence of side effects would be minimised. The observed synergism would also imply that the chance of resistance developing against the oil is reduced.

The combination, however, exhibited indifferent activity against *Candida albicans* ATCC 90028 (FICI: 2.04) suggesting that a combination of the two oils may not be better than the use of the oils as single entities in the management of dermatophytoses such as candidiasis.

5.5 Correlation between inhibition zone diameters and concentration of clove oil.

Log concentration-inhibition zone diameter and absolute concentration-inhibition zone diameter curves were plotted and regression analyses done to establish the relationship between the concentration and the effect of the oil (Fig 1-4).The R^2 values for the log concentration-inhibition zone diameter curves ranged between 0.818 to 0.964 thus fitting well into the linearity curve. The absolute concentration-inhibition zone diameter plot (Curve D1 series 1) showed less correlation (R^2=0.796) compared with the log dose-response plot

(R^2=0.9318) suggesting that in any qualitative determination assays, the log dose-response plot gives better results. This finding is in agreement with the standard method of potency estimation in microbiological assays using log dose-response curve (William and Stephen, 1989).

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

Syzygium aromaticum oil exhibited antimicrobial activity against *Staphylococcus aureus* ATCC 25923 (IZD; 18 mm), MRSA (IZD; 16 mm) and *Escherichia* coli ATCC 25922 (IZD; 18 mm). *Eucalyptus citriodora* oil was also active against *Staphylococcus aureus* ATCC 25923 (IZD; 19 mm), MRSA (IZD; 23 mm) and *Escherichia* coli ATCC 25922 (IZD; 20 mm). *Pseudomonas aeruginosa* was resistant to both Syzygium *aromaticum* and *Eucalyptus citriodora* oils (IZD; 8 mm for each).

Syzygium aromaticum oil had activity against *Microsporum gypseum* (IZD; 11 mm), *Trychophyton mentagrophytes* (IZD; 14 mm), *Candida albicans* ATCC 90028 (IZD; 15 mm) and *Cryptococcus neoformans* (IZD; 10 mm).*Eucalyptus citriodora* oil had activity against *Microsporum gypseum* (IZD; 20 mm), *Trychophyton mentagrophytes* (IZD; 18 mm), *Candida albicans* ATCC 90028 (IZD; 18 mm) and *Cryptococcus neoformans* (IZD; 15 mm).

Syzygium aromaticum oil was effective at lower concentrations against both Staphylococcus *aureus* ATCC25923 (MIC; 1.56% v/v) and *E. coli* ATCC 25922 (MIC; 1.56% v/v) and at moderate concentration against MRSA (MIC; 6.25% v/v). *Eucalyptus citriodora* oil was effective at lower concentrations against Staphylococcus *aureus* ATCC25923 (MIC; 3.125% v/v) and at moderate concentration against *E. coli* ATCC 25922 (MIC; 6.25% v/v) MRSA (MIC; 6.25% v/v).

Syzygium aromaticum oil was effective at the lowest concentrations against *Candida albicans* ATCC 90028 (MIC; 1.56% v/v) and at the highest concentration against *Trychophyton mentagrophytes* (MIC; 12.5% v/v). It exhibited moderate activity against *Cryptococcus neoformans* (MIC; 3.125% v/v) and *Microsporum gypseum* (MIC; 6.25% v/v).

Eucalyptus citriodora oil was effective higher concentrations than for *Syzygium aromaticum* against *Candida albicans* ATCC 90028 (MIC; 12.5% v/v)) and *Microsporum gypseum* (MIC; 12.5% v/v).

. *Syzygium aromaticum* was bactericidal against *Staphylococcus aureus* (MBC; 1.56% v/v) and bacteriostatic against *E. coli* (MBC; 50% v/v). *Eucalyptus citriodora* exhibited bactericidal activity against *Staphylococcus aureus* ATCC25923 (MBC; 6.25% v/v) and MRSA (MBC; 6.25% v/v).

For fungi Syzygium *aromaticum* was fungicidal against *Microsporum gypseum* (MFC; 6.25% v/v), *Trychophyton mentagrophytes* (MFC; 25% v/v) but fungistatic against *Candida*

albicans ATCC 90028 (MFC; 12.5% v/v) and *Cryptococcus neoformans* (MFC; 25% v/v). *Eucalyptus citriodora* oil was fungicidal against *Candida albicans* ATCC 90028 (MFC; 12.5% v/v).

Syzygium aromaticum and *Eucalyptus citriodora* oils acted synergistically against *Staphylococcus aureus* ATCC 25923 (FICI: 0.240), *E. coli* ATCC 25922 (FICI: 0.50) MRSA (FICI: 0.48), and *Microsporum gypseum* (FICI: 0.36).The combination, however, exhibited indifferent activity against *Candida albicans* ATCC 90028 (FICI: 2.04)

6.2 RECOMMENDATION

The data generated shows that *Eucalyptus citriodora* and *Syzygium aromaticum* essential oils are potent and in addition have synergistic effect against most of the test pathogens and could therefore be exploited in the management of skin diseases such as furuncles (boils), impetigo, cellulities and acne and candidiasis.

However stability profile studies of the oils could be undertaken to ascertain the possibility of formulating a stable product that can be clinically used. In addition, focus should be directed towards the continued trend of resistance to a wide range of antimicrobial agents by *Pseudomonas aeruginosa* with a view to coming up with new agents that are effective.

REFERENCES

Abdel-Hameed A (2003). Antimalarial drug resistance in the Eastern Mediterranean Region. *Eastern Mediterranean Health Journal* **9**: 492-508.

Accorsi S, Barnabas GA, Farese P, Padovese V, Terranova M, Racalbuto V and Morrone A (2009). Skin disorders and disease profile of poverty: Analysis of medical records in Tigray, northern Ethiopia, 2005-2007. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **103**: 469-475.

Ahmad, A, Khan, A, Akhtar, F, Yousuf S, Xess I, Khan L and Manzoor N. (2011). Fungicidal activity of thymol and carvacrol by disrupting ergosterol biosynthesis and membrane integrity against Candida. *Eurpean Journal of Clinical Microbiology and Infectious Diseases* **30**: 41–50.

Ajose F (2007). Some Nigerian plants of dermatological importance. *International Journal of Dermatology* **46**: 48-55.

Akiyemi K, Oluwa O and Omomigbehin E (2006). Antimicrobial activity of crude extracts of three medicinal plants used in South West Nigeria folk medicine on some food borne bacterial pathogens. *African Journal of Traditional and Complementary Alternative Medicine* **3**: 13-22.

Al-Bayati FA (2008). Synergistic antibacterial activity between *Thymus vulgaris* and *Pimpinella anisum* essential oils and methanol extracts. *Journal of Ethnopharmacology* **116**: 403-406.

Aliahmadi A, Roghanian R, Emtiazi G and Ghassempour A (2011). A simple method for primary screening of antibacterial peptides in plant seed. *Iranian Journal of Microbiology* **3**: 104–108.

Aligiannis, N., Kalpoutzakis, E., Mitaku, S., and Chinou, I. B. (2001). Composition and antimicrobial activity of the essential oils of two Origanum species. J. Agric. Food Chem. **49**: 4168–4170.

Aljeboury G (2013). *Invitro* antibacterial activity of *Eucalyptus microtheca* and *Cinnamon zeylanicum* crude extracts against *Pseudomonas aeruginosa* isolates. *Medical Journal of Babylon* **10**: 784-793

Aristide L, Ngane R, Kuiate J *et al*, (2011). Antibacterial and antioxidant properties of methanol extracts of the stem bark of *Pteleopsis hylodendion*. *Chemotherapy Research and Practice* 2011: 218750.

Arnold P (2009). Why bacteria stop responding to antibiotic treatment. *Molecular Biology*: 275-292. **(**Published Mar 11, 2009)

Ashour H (2008). Antibacterial, antifungal anticancer activities of volatile oils and extracts from stems, leaves and flowers of *Eucalyptus sideroxylon* and *Eucalyptus torquata. Cancer Biology and Therapy* **7**: 399-403.

Ayoola G, Lawore F, Adelowotan T, Aibinu E, Adenipekun E, Coker B and Odugbemi O (2008). Chemical analysis and antimicrobial activity of the essential oil of *Zyzygium aromaticum*. *African Journal of Microbiology Research* **2**: 162-166.

Babu J, RupaSundari A , Indumathi J, Srujan R and Sravanthi M (2011). Study of the antimicrobial activity and minimum inhibitory concentration of essential oils of spices. *Veterinary World* **4**: 311-316.

Bagamboula C, Uyttendaele M and Debevere J (2004). Inhibitory effect of thyme and basil essential oils, carvacrol, thymol, estragol, linalool and p-cymene towards Shigella sonnei and S. flexneri. *Food Microbiology* **21**: 33–42.

Bari M, Vijay K and Kawamoto S (2008). Antimicrobial activity of cloves and cinnamon extracts against food borne pathogens and spoilage bacteria. *Letter of Applied Microbiology* **72**: 9-21.

Bauer AW, Kirby WM, Sherris JC and Jurck M (1996). Antibiotic susceptibility testing by standard single disc method. *American Journal of Clinical Pathology* **451**: 493-496.

Bax R, Mullan N and Verhoef J (2000). The millennium bugs-the need for and development of new antibacterials. *International Journal of Antimicrobial Agents* **16**: 51-59.

Ben Arfa, A., Combes, S., Preziosi-Belloy, L., Gontard, N., and Chalier, P. (2006). Antimicrobial activity of carvacrol related to its chemical structure. *Letter of Applied Microbiology* **43**: 149–154.

Bernard P (2008). Management of common bacterial infections of the skin. *Current Opinion in Infectious Diseases* **21**: 122-128.

Bennis S, Chami F, Chami N, Bouchikhi T, and Remmal A. (2004). Surface alteration of Saccharomyces cerevisiae induced by thymol and eugenol. *Letter of Applied Microbiology* **38**: 454–458.

Bernard P (2008). Management of common bacterial infections of the skin. *Current Opinion on Infectious Diseases* **21***:* 122-128.

Blum CL, Menzinger S and Genné D (2013). Cellulitis: clinical manifestations and management. Rev Med Suisse 9:1812-1815.

Binder B, Lackner H, Poessl B, Propst E, Weger W, Smolle J and Ginter-Hanselmayer G (2011). Prevalence of *tinea capitis* in Southeastern Austria between 1985 and 2008: up-to-date picture of the current situation. *Mycoses* **54**: 243-247.

Briozzo J, Nunez L, Chirife J, Herszage L and D'Aquino M (1989). Antimicrobial activity of clove oil dispersed in concentrated sugar solution. *The Journal of Applied Bacteriology* **66:** 69-75.

Budak A, Bogusz B, Tokarczyk M and Trojanowska D (2013). Dermatophytes isolated from superficial fungal infections in Krakow, Poland, between 1995 and 2010. *Mycoses* **56**: 422-428.

Burt S and Reiners R (2003). Antibacterial activity of selected plant essential oils against *Escherichia coli* 0157: H 7. *Letters in Applied Microbiology* **36**: 21-67.

Burt S, Van Der Zee R, Koets A, De Graaff A, Van Knapen F, Gaastra W, Haagsman H , and Veldhuizen E (2007). Carvacrol induces heat shock protein 60 and inhibits synthesis of flagellin in Escherichia coli O157:H7. *Applied and Environmental Microbiology* **73**: 4484–4490.

Caballero B, Trugo L, and Finglas P (2003*). Encyclopedia of Food Sciences and Nutrition*. Amsterdam: Academic Press.

Cantrell W and Elewksi B (2014). Can *pityriasis versicolor* be treated with 2% ketoconazole foam? *J Drugs Dermatol.* **13**: 855-859.

Crespo-Erchiga V, Gómez-Moyano E, Crespo M (2008). *Pityriasis versicolor* and the yeasts of genus *Malassezia*. *Actas Dermosifiliogr.* **99**: 764-771.

CLSI (2003). Clinical and Laboratory Standards Institute Quality Manual; Third Edition. Wayne, Pennsylvania. 1908-1898 USA.

Cristani M, D'Arrigo M, Mandalari G, Castelli F, Sarpietro M, Micieli D, Venuti V, Bisignano G, Saija A, and Trombetta D (2007). Interaction of four monoterpenes contained in essential oils with model membranes: implications for their antibacterial activity. *Journal of Agricultural and Food Chemistry* **55**: 6300–6308.

Cook I (2008). Antimicrobial activity of *Eucalyptus major* and *Eucalyptus baileyana* methanolic extracts. *The Internet Journal of Microbiology* Vol. 6 No. 1.

Cowan M (1999). Plant products as antimicrobial agents. *Clinical Microbiology Reviews* **12**: 564-582.

Delaquis P, Stanich K and Mazza G (2002). Antimicrobial activity of individual and mixed fractions of dill, *Coriander* and *Eucalyptus* essential oils. *International Journal of Food Microbiology* **74**: 101-109.

Demos M, McLeod M, Nouri K(2012). Recurrent furunculosis: a review of the literature. *The British Journal of Dermatology* **167**: 725-732.

Demuner A, Barbosa L, Magalhaes, C, Da Silva C, Maltha C and Pinheiro A (2011). Seasonal variation in the chemical composition and antimicrobial activity of volatile oils of three species of Leptospermum (Myrtaceae) grown in Brazil. *Molecules* **16**: 1181–1191.

Dickson R. Ekuadzi E, Annan K, and Komlaga G. (2011). Antibacterial, anti-inflammatory, and antioxidant effects of the leaves and stem bark of *Glyphaea brevis* (Spreng) Monachino (*Tiliaceae):* A comparative study. *Pharmacognosy Research* **3:** 166–172.

Di Pasqua R, Betts G, Hoskins N, Edwards M, Ercolini D and Mauriello G (2007). Membrane toxicity of antimicrobial compounds from essential oils. *Journal of Agricultural and Food Chemistry* **55**: 4863–4870.

Djenane D, Yangüela J, Montañés L, Djerbal M and Roncalés P (2011). Antimicrobial activity of *Pistacia lentiscus* and *Satureja montana* essential oils against *Listeria monocytogenes* CECT 935 using laboratory media: efficacy and synergistic potential in minced beef. *Food Control* **22**: 1046–1053.

Domadia P, Swarup S, Bhunia A, Sivaraman J and Dasgupta D (2007). Inhibition of bacterial cell division protein FtsZ by cinnamaldehyde. *Biochemical Pharmacology* **74**: 831–840.

. Dorman H and Deans G (2005). Antimicobial agents from plants: Antibacterial activity of volatile oils. *Journal of Applied Microbiology* **88**: 308-316.

Domenico et al, (2005). Mechanisms of antibacterial actions of three monoterpenes. *Antibacterial Agents and Chemotheraphy* **49**: 2474-2478.

Dykes, Turner M, Caffin N, Weerakkhody N and Lambert L (2011). Synergistic antimicrobial activity of *Alphinia galangal*, *Rosmarinus officinalis* and *Eucalyptus staigerrana* extracts. *Journal of the Science of Food and Agriculture* **91**: 461- 468.

El-Gohary M, van Zuuren E, Fedorowicz Z, Burgess H, Doney L, Stuart B, Moore M and Little P (2014). Topical antifungal treatments for *tinea cruris* and *tinea corporis*. *Cochrane Database Systematic Review*8:CD009992.doi: 10.1002/14651858.CD009992.pub2.

Eliopoulos G and Moellering R (1982). Antibiotic synergism and antimicrobial combinations in clinical infections. *Reviews of Infectious Diseases* **4**: 282-293.

Espina L, Somolinos M., Lorán S, Conchello P, García D and Pagán R (2011). Chemical composition of commercial citrus fruit essential oils and evaluation of their antimicrobial activity acting alone or in combined processes. *Food Control* **22**: 896–902.

Eugénia P, Luís V, Carlos C *et al* (2009). Antifungal activity of the clove essential oil from *Syzygium aromaticum* on *Candida*, *Aspergillus* and dermatophyte species. *Journal of Medical Microbiology* **58**: 1454-1462.

Fankham et al (2011). Antibacterial activities of selected Cameroonian species and their synergistic effects with antibiotics against multi-drug resistant phenotypes. *BMC Complementary and alternative Medicine* Nov 1: 11: 104. Doi: 10. 1186/ 1472-688211-104.

Fathy H, El-Mongy S, Baker N, Abdel-Azim Z and El-Gilany A (2004). Prevalence of skin diseases among students with disabilities in Mansoura, Egypt. *Eastern Mediterranean Health Journal* **10**: 416-424.

Fernandes G, de Mesquita R, Randau K, Franchitti A and Ximenes E (2012). *In-vitro* synergistic effect of *Psidium guineense* in combination with antimicrobial agents against MRSA strains. *The Scientific World Journal* 2012 DOI: 1100/ 2012/ 158237. Epub 2012 Apr 24.

Fernandez R, Tubau F, Ariza J, Gudiol F, Cabellos C, Dominguez M, Ribes S and Pachon-Ibanez M (2011). Efficacy of fosfomycin and its combination with linezolid, vancomycin and imipenem in an experimental peritonitis model caused by a *Staphylococcus aureus* strain with reduced susceptibility to vancomycin. *European Journal of Clinical Microbiology and Infectious Diseases* **30**: 89-95.

Feron V, Til H, De Vrijer F, Woutersen R, Cassee F and Van Bladeren P (1991). Aldehydes: occurrence, carcinogenic potential, mechanism of action and risk assessment. *Mutat. Res.* **259**: 363–385.

Finch R and Hunter PA (2006). Antibiotic resistance--action to promote new technologies: Report of an EU Intergovernmental Conference held in Birmingham, UK, 12-13 December 2005. *Journal of Antimicrobial Chemotherapy* **58**: 13-122.

Fitzgerald D, Stratford M, Gasson M , Ueckert J, Bos A and Narbad A (2004). Mode of antimicrobial of vanillin against *Escherichia coli*, *Lactobacillus plantarum* and *Listeria innocua*. *Journal of Applied Microbiology* **97**: 104–113.

Fitzgerald D, Stratford M, Gasson M and Narbad A (2005). Structure-function analysis of the vanillin molecule and its antifungal properties. *Journal of Agricultural and Food Chemistry* **53**: 1769–1775.

Fullerton M, Khatiwada J, Johnson J, Davis S and Williams L (2011). Determination of antimicrobial activity of Sorrel on *Escherichia coli* 0157: H 7 isolated from food veterinary and clinical samples. *Journal of Medicinal Food* **14:** 950-956.

Gabel C and Berg H (2003). The speed of the flagellar rotary motor of *Escherichia coli* varies linearly with protonmotive force. *Proceedings of the National Academy of Sciences U.S.A.* **100**: 8748–8751.

Gabillot-Carré M and Roujeau JC (2007). Acute bacterial skin infections and cellulitis. *Current Opinion on Infectious Diseases* **20**: 118-123.

George A and Rubin G (2003). A systematic review and meta-analysis of treatments for impetigo. *British Journal of General Practice* **53**: 480-487.

Gill A and Holley R (2006a). Disruption of *Escherichia coli, Listeria monocytogenes and Lactobacillus sakei* cellular membranes by plant oil aromatics. *International Journal of Food Microbiology* **108**: 1–9.

Gill A and Holley R (2006b). Inhibition of membrane bound ATPases of *Escherichia coli* and *Listeria monocytogenes* by plant oil aromatics. *International Journal of Food Microbiology* **111**: 170-174.

Ginter-Hanselmayer G, Weger W, Ilkit M and Smolle J (2007). Epidemiology of tinea capitis in Europe: current state and changing patterns. *Mycoses* 50: 6-13.

Gorwitz RJ (2008). A review of community-associated methicillin-resistant *Staphylococcus aureus* skin and soft tissue infections. *Pediatric Infectious Disease Journal* **27**: 1-7.

Gunderson C (2011). Cellulitis: definition, etiology, and clinical features. **Am J Med. 124:** 1113-1122.

Gupta C, Amar P, Ramesh C and Kumari A (2008). Antibacterial activity of some herbal oils against common food borne pathogens. *African Journal of Microbiology Research* **2**: 258-261.

Gupta A and Lyons D (2014). *Pityriasis versicolor*: an update on pharmacological treatment options. *Expert Opin Pharmacother* 15: 1707-1713.

Hackett B, O'Connell K, Cafferkey M, O'Donnell B and Keane FM. *Tinea capitis* in a paediatric population. Ir Med J. 99: 294-295.

Hammer K, Carson C and Riley T (1999). Antimicrobial activity of essential oils and other extracts. *Journal of Applied Microbiology* **86**: 985-990.

Heard J, McDonald K, Xing Y, Kluesner K, Liao J and Wibbenmeyer L (2014). Regional and National Review of Factors Associated With Burn Wound Cellulitis. J Burn Care Res. 2014 Aug 6. [Epub ahead of print].

Havlickova B, Czaika V and Friedrich M (2008). Epidemiological trends in skin mycoses worldwide. *Mycoses* **4**: 2-15

Helander I, Alakomi H, Latva-Kala K, Mattila-Sandholm T, Pol I, Smid E, Gorris L, and Von Wright A. (1998). Characterization of the action of selected essential oil components on Gram-negative bacteria. *Journal of Agricultural and Food Chemisry* **46**: 3590–3595.

Hemaiswarya D and Doble M (2009). Synergistic interaction of eugenol with antibiotics against Gram negative bacteria. *International Journal of Phytotherapy and Phytopharmacology* **16**: 997-1005.

Hemaiswarya S, Soudaminikkutty R, Narasumani M, and Doble M (2011). Phenylpropanoids inhibit protofilament formation of Escherichia coli cell division protein FtsZ. *Journal of Medical Microbiology* **60**: 1317–1325.

Henry E, Worthington T, Conway B and Lambert P (2009). Antimicrobial efficacy of *Eucalyptus oil* and 1,8-cineole alone and in combination with chlorhexidine digluconate against microorganisms grown in planktonic and biofilm. *Journal of Antimicrobial Chemotherapy* **64**: 1219-1225.

Hyldgaard M, Mygid T and Meyer R (2012). Essential oils in food preservation. *Frontiers in Microbiology* 2012; 3:12. Epub 2012 Jan 25.

Javad S and Atefeh A (2010). Antimicrobial and antifungal properties of the essential oil and methanol extracts of *Eucalyptus largiflorens* and *Eucalyptus intertexta. Pharmacognosy Magazine* **6**: 172-175.

Javad S and Batooli H (2010). Chemical composition and antimicrobial activity of the volatile oil of *Eucalyptus sargentii maiden* cultivated in central Iran. *International Journal of Green Pharmacy* **4**: 174-177.

Johnston PG and Longley DB (2005). Molecular mechanisms of drug resistance. *Journal of Pathology* **2**: 275-292.

Jones T, Jarratt M, Mendez-Moguel I, Paz N, Grekin S, Cognata Smith C and Kaur M (2014). A randomized, multicenter, double-blind, vehicle-controlled study evaluating the efficacy and safety of luliconazole cream 1% once daily for 7 days in patients aged ≥ 12 years with tinea cruris. ***J Drugs Dermatol.*** 13: 32-38.

Juliani R and Bassol I (2012). Essential oils in combination and their antimicrobial properties. *Molecules* **17**: 3989-4006.

Juven B, Kanner J, Schved F and Weisslowicz H (1994). Factors that interact with the antibacterial action of thyme essential oil and its active constituents. *Journal of Applied Bacteriology* **76**: 626–631.

Jung H and Fahey G (1983). Nutritional implications of phenolic monomers and lignin: a review. *Journal of Animal Science* **57**: 206–219.

Kalemba D and Kunicka A (2003). Antibacterial and antifungal properties of essential oils. *Current Medical Chemistry* **10**: 813-829.

Kamal R and Radhika J (2010). Antibacterial activity of *Syzygium aromaticum* and its bud oil against dental carries causing microorganisms. *Ethnobotanical Leaflets* **14**: 960-975.

Komba EV and Mgonda YM (2010). The spectrum of dermatological disorders among primary school children in Dar es Salaam. *BMC Public Health* **10**: 765 doi: 10.1186/1471-2458-10-765

Konate K, Hilou A, Barro N, Mavoungou J, Lepengue A, Souza A, Datte J, Mbatchi B and Nacoulma O (2012). Antimicrobial activity of polyphenol-rich fractions from *Sidaaiba L* against Cotrimoxaxole-resistant bacteria strains. *Annals of Clinical Microbiology and Antimicrobials* 2012 Feb 24;11:5.

Koning S, van der Sande R, Verhagen A, van Suijlekom-Smit L, Morris A, Butler C, Berger M and van der Wouden J (2012). Interventions for impetigo. *Cochrane Database Systematic Review* 18; 1:CD003261. doi: 10.1002/14651858.CD003261.pub3.

Labia R, Barthelemy M, Peduzzi J, Morand A, Tiwari K and Kazmierczak A (1990). Overcoming enzymatic resistance in bacteria: Impact on future therapy. *The Journal of International Medical Research* 4: 48D-57D

Lacmata S, Kuete V, Dzoyem P, Tankeo S, Teke G, Kuiate J and Pages J (2012). Antibacterial activities of selected Cameroonian plants and their synergistic effects with a antibiotics against bacteria expressing MDR phenotypes. *Evidence-Based Complimentary and Alternative Medicine* 2012: 623723. doi: 10.1155/2012/623723. Epub 2012 Feb 28.

Laekeman G, Van Hoof L, Haemers A, Berghe D, Herman A and Vlietinck A (1990). Eugenol a valuable compound for in vitro experimental research and worthwhile for further in vivo investigation. *Phytotherapy Research* **4**: 90–96.

Lambert R, Skandamis P, Coote P and Nychas G (2001). A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *Journal of Applied Microbiology* **91**: 453–462.

Lambert P, Karpanen T, Worthington T, Hendry R and Conway R (2008). Antimicrobial efficacy of chlorhexidine digluconate alone and in combination with *Eucalyptus* oil, tea

tree oil and thymol against planktonic and biofilm cultures of *Staphylococcus epidermidis. Journal of Antimicrobial Chemotherapy* **62**: 1031-1036.

La Storia A, Ercolini D, Marinello F, Di Pasqua R, Villani F and Mauriello G (2011). Atomic force microscopy analysis shows surface structure changes in carvacrol-treated bacterial cells. *Research in Microbiology* **162**: 164–172.

Laube S (2004). Skin infections and ageing. *Ageing Research Reviews* **3**: 69-89.

Lertzman B and Gaspari A (1996). Drug treatment of skin and soft tissue infections in elderly long-term care residence. *Drugs and Aging* **9**: 109-121.

Lugman S, Darokar P, Kalra A and Khanuja S (2008). Antimicrobial activity of *Eucalyptus citriodora* essential oil. *International Journal of Essential Oil Therapeutics* 2: 69-75.

Lyakhovitsky A, Shemer A and Amichai B (2013). Molecular analysis of *Malassezia* species isolated from Israeli patients with *pityriasis versicolor*. *International Journal of Dermatology* **52**: 231-233.

Mahmoud A and Louis B (1999). Correlation of These Mechanisms with Bacterial Resistance. *Clinical Microbiology Reviews* **4:** 501–517

Mann C, Cox S, and Markham J (2000). The outer membrane of Pseudomonas aeruginosa NCTC 6749 contributes to its tolerance to the essential oil of Melaleuca alternifolia (tea tree oil). *Letters in Applied Microbiology* **30**: 294–297.

Maria A., Athanasios A, George P and Dimitrios L (2003). Effect of polyamines on the inhibition of peptidyltransferase by antibiotics: revisiting the mechanism of chloramphenicol action.*Nucleic Acids Research* **31**: 5074-5083

Marrone R, Vignally P, Rosso A, Didero D, Pizzini E, Dassoni F, Pajno M, Maiani E, Franco G, Calcaterra R, Morrone A and Mirisola C (2012). Epidemiology of skin disorders in Ethiopian children and adolescents: An analysis of records from the Italian dermatological centre, Mekelle, Tigray, Ethiopia, 2005 to 2009. *Paediatric Dermatology* doi: 10.111/j. 525-1470.2011.01698.x.

Mathew E (2004). Pharmacodynamics of antimicrobial drugs. *Diseases of North America* **18**: 451-465.

Mathews H (2009). Clove oil effective in *Candida albicans* treatment. *The Environmental Illness Resource*. Thursday July 2009 **20**: 17.

Mayaud L, Carricajo A, Zhiri A and Aubert G (2008). Comparison of bacteriostatic and bactericidal activity of 13 essential oils against strains with varying sensitivity to antibiotics. *Letters in Applied Microbiology* **47**: 167-173.

Mbah J, Ngemenya M, Pierre T and Titanji V (2006). Antibacterial effects of some Cameroonian medicinal plants against common pathogenic bacteria. *African Journal of Traditional, Complementary and Alternative* M*edicine* **3**: 84-93.

Moellering R (1983). Rationale for use of antimicrobial combinations. *American Journal of Medicine* **75**: 4-8.

Moshi J, Mbwambo H Omar J and Van den J (2007). Brine shrimp toxicity evaluation of some Tanzanian plants used traditionally for the treatment of fungal infections. *African Journal of Traditional, Complementary and Alternative Medicine* **4**: 219-225.

Moulin F, Quinet B, Raymond J, Gillet Y and Cohen R (2008). Managing children skin and soft tissue infections. *Archives of paediatrics* **2**: 62-67.

Musa N, Seongwei L, Wee W, Musa N (2009). Chemical composition and antimicrobial activity of the essential oil of *Syzygium aromaticum* flower bud (Clove) against fish systemic bacteria isolated from aquaculture sites. *Frontiers of Agriculture in China* **3**: 332-336.

Nannapaneni R, Chalova V, Crandall P, Ricke S, Johnson M and O'Bryan C (2009). Campylobacter and Arcobacter species sensitivity to commercial orange oil fractions. *International Journal of Food and Microbiology* **129**: 43–49.

Nayan R and Shukla V (2011). Antibacterial and antifungal activities from leaf extracts of Cassia fistula 1. An ethnomedicinal; plant. *Journal of Advanced Pharmaceutical Technology and Research* **2**: 104-109.

Naseri A, Fata A, Najafzadeh M and Shokri H (2013). Surveillance of dermatophytosis in northeast of Iran (Mashhad) and review of published studies. **Mycopathologia. 176:** 247-253.

Nikaido H. (2003). Molecular basis of bacterial outer membrane permeability revisited. Microbiology and Molecular *Biology Reviews* **67**: 593–656.

Njume C, Afolah J and Ndip R (2011). Inhibitory and bactericidal potential of crude acetone extracts of *Combretum molle* on drug resistant strains of *Helicobacter pylori*. *Journal of Health, Population and Nutrition* **29**: 438-445.

Norrby S, Nord C and Finch R (2005). Development of new antimicrobial drugs: A potentially serious threat to public health. *Lancet Infectious Disease* **5**: 115-119.

Okoye T, Akah P, Okoli C, Ezike C, Omeje E and Odoh E (2012). Antimicrobial effects of lipophylic fraction and kaurenoic acid isolated from the root bark extracts of *Annonas senegalensis. Evidence-based Comlementary and Alternative Medicine*. May 24 doi: 10.1155/ 2012/831327

Osanaiye A, Agbaji A and Dakare M (2007). Antimicrobial activity of oils and extracts of *Cymbopogon citrus*, *Eucalyptus* citriodora and *Eucalyptus camaldulensis*. *Journal of Medicinal Science* **7**: 694-697.

Ouellette M (2001). Drug resistance-antibiotic or antimicrobial resistance. *Tropical Medicine and International Health* **6***:* 874-882.

Paibon W, Yimnoi C, Tembab N, Boonlue W, Jampachaisri K, Nuengchamnong N, Waranuch N and Ingkaninan K (2011). Comparison and evaluation of volatile oils from three different extraction methods for some Thai fragrant flowers. *International Journal of Cosmetic Science* **33**:150–156.

Pankaj B, Nayan R, Shukla V and Achaya R (2011). Antimicrobial and antifungal activities of *Cordia dichotoma* bark extracts. *An International Quaterly Journal of Research in Ayurveda* **32**: 585-589.

Park M, Gwak K, Yang F *et al* (2007). Antifungal activities of the essential oils in *Syzygium aromaticum* and *Leptospermum petersonii* and their constituents against various dermatophytes. *Journal of Microbiology* **45**: 460-465.

Pauli A and Kubeczka K (2010). Antimicrobial properties of volatile phenylpropanes. *Natural Product Communications* **5**: 1387–1394.

Pereira C and Meireles M (2010). Supercritical fluid extraction of bioactive compounds: fundamentals, applications and economic perspectives. *Food and Bioprocess Technology* **3**: 340–372.

Pinto E, Luis U, Carlos C and Ligas S (2009). Antifungal activity of the clove essential species. *Medicinal Microbiology* **58**: 1454-1462.

Powers JH (2003). Development of drugs for antimicrobial-resistant pathogens. *Current Opinion on Infectious Diseases* **16**: 547-551.

Raghavan M and Linden P (2004). Newer treatment options for skin and soft tissue infections. *Drugs* **64**: 1621-1642.

Raghu Prasada Malladar Shivamurthy, Shashikala Gowdara Hanumantappa Reddy, Ravindra Kallappa, Shankar Achar Somashekar, Deepa Patil and Umakant N Patil (2014).

Comparison of Topical Anti- Fungal Agents Sertaconazole and Clotrimazole in the Treatment of *Tinea Corporis*-An Observational Study. ***J Clin Diagn Res.*** **8**: HC09–HC12.

Rao A, Zhang Y, Muend S and Rao R (2010). Mechanism of antifungal activity of terpenoid phenols resembles calcium stress and inhibition of the TOR pathway. *Antimicrobial Agents and Chemotherapy* **54**: 5062–5069.

Rasooli L, Shayegh S and Astaneh S (2009). The effect of *Menta spicata* and *Eucalyptus camaldulensis* essential oils on dental hygiene. *International Journal of Dental Hygiene* **7**: 196-203.

Rattanachaikunsopon P and Phumkhachorn P (2010). Assessment of factors influencing antimicrobial activity of carvacrol and cymene against *Vibrio cholerae* in food. *Journal of Bioscience and Bioengeneering* **110**: 614–619

Rhayour K, Bouchikhi T, Tantaoui-Elaraki A, Sendide K and Remmal A (2003). The mechanism of bactericidal action of oregano and clove essential oils and of their phenolic major components on Escherichia coli and Bacillus subtilis. *Journal of Essential Oil Research* **15**: 356–362.

Richard D and Joanna C (2002). Antimicrobial resistance: A global response. *Bullettin of the World Health Organization* **80**: 126-133.

Roller S (2003). Natural Antimicrobials for the Minimal Processing of Foods. Cambridge: Woodhead Publishing, Limited.

Salari M, Amine G, Shiraz M, Hafezi R and Mohammadypour M (2006). Antibacterial effects of *Eucalyptus globules* leaf extract on pathogenic bacteria isolated from specimens with respiratory tract disorders. *Clinical Microbiology and Infection* **12**: 194-196.

Sánchez E, García S and Heredia, N. (2010). Extracts of edible and medicinal plants damage membranes of Vibrio cholerae. *Applied and Environmental Microbiology* **76**: 6888–6894.

Sean D, Cindy M, Julie L, John E. John R and Wyllie S (2001). Determining the antimicrobial actions of tea tree oil. *Molecules* **6**: 87-91.

Seenivasan P, Manickkam J and Savarimuthu I (2006). *In vitro* antibacterial activity of some plant essential oils. *BMC Complementary and Alternative Medicine* 6:39 doi:10.1186/1472-6882-6-39.

Senthil N (2007). The use of *Eucalyptus tereticornis* oil as a natural larvicidal against the malaria vector *Anopheles stephensi. Linton-Bioresource Technology* **98**: 1856-1860.

Shapira R and Mimran E (2007). Isolation and characterization of Escherichia coli mutants exhibiting altered response to thymol. *Microbial Drug Resistance* **13**: 157–165.

Sharma S and Verma K (2001). Skin and soft tissue infection. *Indian Journal of Pediatrics* **3**: 546-550.

Sheldon A Jr (2003). Antibiotic resistance: Who is winning the war? Introductory remarks. *International Journal of Toxicology **22***: 129-130.

Sing H, Mittal S, Kaur S, Batis D and Kohli R (2009). Characterization and anti-oxidant activity of essential oils from fresh and decaying leaves of *Eucalyptus tereticornis*. *Journal of Agricultural and Food Chemistry* **57**: 6962-6966.

Sippe JR (1991). Common skin infections. *Australian Family Physician* **20:** 783-784.

Sladden M and Johnston G (2005). Current options for the treatment of impetigo in children. Expert Opin Pharmacother **6**: 2245-2256.

Sporer F, Mulyaningsih S, Reichling J and Wink M (2011). Antibacterial activity of essential oils from *Eucalyptus* and selected components against multi-drug resistant bacterial pathogens. *Pharmaceutical Biology* **49**: 893-899.

Springer I, Russo P, Wiltfang J, Wiltfang J, Fickenscher H, Sherry E, Warnke P, Becker S, Podschun R and Sivananthan S (2009). The battle against multi-drug resistant strains: Renaisance of antimicrobial essential oils as a promising force to fight hospital-acquired infections. *Journal of Cranio-Maxillo-Facial Surgery* **37**: 392-397.

Stulberg D, Penrod M and Blatny R (2002). Common bacterial skin infections. *American Family Physician* **66**: 119-124.

Sudsadu V, Panuwat P and Tunyarut J (2010). Antimicrobial effects of coating solution containing clove oil and hydrophobic starch for coating paperboard. *Asian Journal of Food and Agro-Industry* **3**: 204-212.

Suliman S, Vuuren F and Viljoen M (2009). The antimicrobial activity of four commercial essential oils in combination with conventional antimicrobials. *Letters in Applied Microbiology* **48**: 440-446.

Sun S, Fu Y, Zu Chen L, Shi Y, Wang Z and Efferth T (2007). Antimicrobial activity of clove and rosemary essential oil alone and in combination. *Phytotherapy Research* **21**: 989-994.

Surendran K, Bhat RM, Boloor R Nandakishore B and Sukumar D (2014). A clinical and mycological study of dermatophytic infections. *Indian Journal of Dermatology* **59**: 262-267.

Takahashi T, Kokubo R and Sakaino M (2004). Antimicrobial activities of *Eucalyptus* leaf extracts and flavanoids from *Eucalyptus maculate. Letters of Applied Microbiology* **39**: 60-64.

Tan H, Tay Y and Goh C (1998). Bacterial skin infections at a tertiary dermatological centre. *Singapore Medical Journal* **39**: 353-356.

Tatjana M , Dragan R *et al* (2010). Antimicrobial activity of *Satureja Hortensis* L. essential oil against pathogenic microbial strains. *Archives of Biological Sciences Belgrade* **62**: 159-166.

Tiwar T, Bharti S, Kaur D, Dikshit R and Hoondal G (2005). Synergistic antibacterial activity of tea and antibiotics. *The Indian Journal of Medical Research* **122**: 80-84.

Tohidpour A, Sattari M, Omidbalgi R, Yadegar A and Nazemi J (2010). Antibacterial effect of essential oils from two medicinal plants against methicillin-resistant *Staphylococcus aureus* (MRSA). *Phytomedicine; International Journal of Phytotherapy and Phytopharmacology* **17**: 142-145.

Thiers BH (1998). Dermatology therapy update. *Medical Clinics of North America* **82**: 1405-1414.

Tiago F, Amanda R, Carneiro de Mesquita, Karina R, Adelisa F, and Eulalia A (2012). *In vitro* synergistic effect of *Psidium guineense* (Swartz) in combination with antimicrobial agents against methicillin-resistant *Staphylococcus aureus* strains. *Scientific World Journal* 2012: 158237.

Trivedi N and Hotchandani S (2004). A study of the antimicrobial activity of oil of *Eucalyptus*. *Indian Journal of Pharmacology* **36**: 93-94.

Triviño-Duran L, Torres-Rodriguez J, Martinez-Roig A, Cortina C, Belver V, Perez-Gonzalez M and Jansa J (2005). Prevalence of tinea capitis and tinea pedis in Barcelona schoolchildren. *Pediatric Infectious Disease Journal* **24**: 137-141.

Trombetta D, Castelli F, Maria G, Vincenza V, Cristani M, Daniele C, Antonella S, Gabriela M and Giuseppe B (2005). Mechanisms of antibacterial action of three monoterpenes. *Antimicrobial Agents and Chemotheraphy* **49**: 2474–2478.

Turina A, Nolan M, Zygadlo J and Perillo M (2006). Natural terpenes: self-assembly and membrane partitioning. *Biophysical Chemistry* **122**: 101–113.

Ultee A, Bennik M and Moezelaar R (2002). The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen Bacillus cereus. *Applied and Environmental Microbiology* **68**: 1561–1568.

van Zuuren E, Fedorowicz Z, El-Gohary M (2014). Evidence based topical treatments for tinea cruris and tinea corporis: A summary of a Cochrane systematic review. *British Journal of Dermatology* doi: 10.1111/bjd.13441. [Epub ahead of print].

Veldhuizen E, Tjeerdsma-Van Bokhoven J, Zweijtzer C, Burt S and Haagsman H (2006). Structural requirements for the antimicrobial activity of carvacrol. *Journal of Agricultural and Food Chemistry* **54**: 1874–1879.

Vingsbo Lundberg C and Frimodt-Møller N (2003). Efficacy of topical and systemic antibiotic treatment of meticillin-resistant *Staphylococcus aureus* in a murine superficial skin wound infection model. *International Journal of Antimicrobial Agents* **42**: 272-275.

Vuran E, Karaarslan A, Karasartova D, Turegun B and Sahin F (2014). Identification of *Malassezia* species from *pityriasis versicolor* lesions with a new multiplex PCR method. *Mycopathologia* **177:** 41-49.

Walsh S, Maillard J, Russeli A, Catrenich C, Charbonneau D and Bartolo R (2003). Activity and mechanisms of action of selected biocidal agents on Gram-positive and – negative bacteria. *Journal of Applied Microbiology* **94**: 240-247.

Wiese-Posselt M, Heuck D, Draeger A, Mielke M, Witte W, Ammon A, Hamouda O (2007). Successful termination of a furunculosis outbreak due to lukS-lukF-positive, methicillin-susceptible *Staphylococcus aureus* in a German village by stringent decolonization, 2002-2005. *Clinical Infectious Disease* **44**: 88-95.

Weissmann A (1989). Infectious diseases of the skin. *Geriatrics* **44**: 20-22.

Yamasaki M, Tamura N, Nakamura K, Sasaki N, Murakami M, Rajapakshage W, Kumara B, Tamura Y, Lim S, Ohta H, Takiguchi M (2011). Effects and mechanisms of action of polyene macrolide antibiotic nystatin on Babesia gibsoni in vitro. *The Journal of Parasitology* **6:** 1190-1192

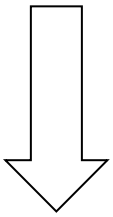
Yang A and Kerdel F (2006). Infectious disease update: New anti-microbials. *Seminars in Cutaneous Medicine and Surgery* **25**: 94-99.

Xu J, Zhou F, Ji B, Pei R and Xu N (2008). The antibacterial mechanism of carvacrol and thymol against Escherichia coli. *Letters in Applied Microbiology* **47**: 174–179.

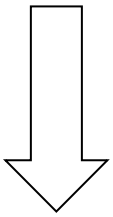
APPENDICES

APPENDIX 1

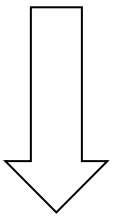
Initial screening of *Eucalyptus* and clove oils for antimicrobial activity when acting singly



Screening of antimicrobial activity of *Eucalyptus* and clove oils when combined.



Determination of minimum inhibitory concentrations of *Eucalyptus* and clove oils when acting singly (Fungal and Bacterial)



Determination of minimum inhibitory concentrations of *Eucalyptus* and clove oils when combined (Fungal and Bacterial)

APPENDIX 2

ganisms	**ne of inhibition (mm)**		**alue1**	**D**	**alue2**	**alue3**
	ove oil ean (SD)	**calyptus oil** ean (SD)		**Combine d**		
phylococcus aureus	(2.2)	(`1.9)	47	(0.1)	01	57
cherichia coli	(2.0)	(1.9)	81	(0.3)	36	22
ethicillin resistant *staphylococcus aureus*	(2.1)	(1.9)	13	(0.3)	84	06
eudomonas aeruginosa	1.9)	2.0)	69			
crosporum gypseum	(2.4)	(2.0)	08	(0.3)	07	19
chophyton mentagrophytes	(2.2)	(1.8)	80			
ndida albicans	(2.0)	(2.0)	62	(0.4)	95	37
yptococcus neoformans	(2.0)	(0.4)	13			

[1]*Syzygium aromaticum* (clove) vs *Eucalyptus citriodora*, [2]*Syzygium aromaticum* vs Combined, [3] *Eucalyptus citriodora* vs combined

n/d..... not done