Studies on the Effects of Herbs and Herbal Extracts on Bacteria Notably MRSA

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In the name of Allah, most gracious, most merciful

Dedication

To my parents, to my husband Mohammed, to my children (Nawaf, Suod, Naif, Talal and Ahmed) and to my family.

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Abstract

As a result of the emergence of multi- drug resistance, notably in relation to methicillin-resistant *Staphylococcus aureus* (MRSA), there is an urgent need for effective alternatives to conventional antimicrobial therapy. The overall aim of the work presented here was to investigate the antibacterial properties of some plant extracts as alternatives to antibiotics. Particular attention is paid here to herbal therapy, aromatherapy and combination therapy and an evaluation was made of the *in vitro* antibacterial activities of nineteen plant extracts against Gram-negative bacteria and Gram-positive bacteria including MRSA and two species of yeast (*Candida rugosa* and *Candida inconspicua*). The antimicrobial activity of each plant extract was evaluated alone and in a variety of combinations (two plant products, with honey and UV therapy). Data were obtained from an agar diffusion assay, minimum inhibitory concentration (MIC), minimum fungicidal/ bactericidal concentration (MFC)/ (MBC) values. The agar diffusion assay was used to assess the effect of combining plant extracts and UVB at different time periods. The antibacterial activity of herbal extractions after being autoclaved also was evaluated. The effect of combining antibiotics including vancomycin 30 µg, ampicillin 10 µg, erythromycin 15 µg, chloramphenicol 30 µg, gentamicin 10 µg and tetracycline 30 µg and plant

extracts was further investigated using the agar disc diffusion method, as was the liberation of endotoxin after being treated with herbal extracts using the Gel-Clot Assay. Out of nineteen plant extracts tested, five essential oils and five herbal extracts showed antibacterial activity against almost all of the microorganisms studied. Kanuka oil, Eucalyptus oil, Tea Tree oil, Goldenseal and Grapefruit seed extract exhibited the greatest antibacterial activity. The antibacterial properties of plant extracts showed a significant increase in the inhibition zone following UV-B exposure compared with the inhibition zone of plant extracts alone. While, some combinations of plant extracts produced a synergistic effect against microorganisms; the combinations between Goldenseal extract (DL) and Eucalyptus oil produced a synergistic effect against *Candida rugosa* and *Candida inconspicua;* this synergistic effect was also seen with combined Goldenseal extract (DL) and Peppermint oil against *Candida inconspicua.* There is a significant difference between autoclaved and non-autoclaved activities for Kanuka oil and Eucalyptus oil against MRSA. Plant extracts also improved the efficacy of almost all antibiotics. In one out of ten cases, only Tea Tree oil released endotoxin from *E.coli.* It is therefore suggested that the use of these plant extracts could be a novel way of combatting bacterial resistance to antibiotics.

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Chapter One: General Introduction

1. Introduction

Since the serendipitous discovery of penicillin in 1928 by Alexander Fleming, antibiotics have played a significant role in the treatment of both bacterial and fungal diseases and have saved millions of lives. Problems with antibiotic resistance are now frequently appearing, however, particularly in relation to methicillin-resistant *Staphylococcus aureus* (MRSA). There is therefore an obvious need for the development of therapeutic alternatives to antibiotics, a reality that has attracted considerable interest as well as concern amongst healthcare and medical practitioners. A number of alternatives to antibiotics are now in common use for the treatment of wounds, including maggot debridement therapy and honey (apitherapy) (Al- Naama, 2009). The aim of the work described in this Thesis was to investigate the antibacterial properties of a variety of herbs and their extracts as a prelude to their possible use in wound treatment. Plants contain a number of components and secondary metabolites that contribute to their antimicrobial activity. Herbal medicines are often safer than synthetic chemical ones and are often inexpensive and more locally available (Bisht *et al*., 2009; Pal and Shukla, 2003).

1.1 Alternative approaches to antibiotic treatment

1.1.1 Herbal therapy

Medicinal plant therapy employs plants (or extracts) for treating infections and illnesses in general (Nwachukwu *et al.,* 2010). Between a quarter, to half a million species of plants have been listed, but only 1% to 10% of these are used by humans and animals as foods and in medicine (Cowan, 1999). For millennia, herbs and herbal extractions have played an important role in healthcare systems throughout the world and amongst nearly all cultures. There is evidence, for example, that Neanderthals living in Iraq, used herbs like hollyhock, as a medical herb, a usage which continues today globally (Ciocan and Bara, 2007; Choudhary *et al*., 2009). In addition, some 5000 years ago, the Chinese, Indians, Egyptians, Syrians Greeks, and Romans depended on herbal medicines (Pal and Shukla, 2003). Rivera *et al.* (2013) reported that Chinese and Indian cultures still often rely upon herbal medicines as an essential component in their treatment. Around 300-400 medicinal plants are mentioned by Hippocrates in the late fifth century B.C. (Cowan, 1999) and approximately 30 healing plants are referred to in the Bible (Cowan, 1999), while at least nineteen medicinal herbs are referenced in the Holy Quran, written 1400 years ago, including, athel tamarisk, date palm, fig, ginger, garlic, lentil, olive, grape, mustard, onion, pomegranate,

sweet basil, the toothbrush tree arak, camphor, summer squash, acacia, cucumber, leek, and cedrus (Azarpour *et al.,* 2014); some of these plants have been recently evaluated in modern, evidence based research (Rivera *et al.,* 2013; Berlin, 2001). Herbal antibiotics are increasingly being used in some developed countries, including the US, where they are distributed and regulated in the form of dietary supplements (Ciocan and Bara, 2007; Bisht *et al*., 2009). Currently, 25% to 50% of all pharmaceutical products used are derived from fungal and bacterial materials (Ciocan and Bara, 2007; Saga and Yamaguchi, 2009) and following the widespread availability of antibiotics, there has been a decline in the use of plants as antimicrobials (Ciocan and Bara, 2007). Interest in natural products is developing worldwide largely because of the increasing development of antibiotic resistance (Sibanda and Okoh, 2007) and over 64 plants have recently been reported to have major antibacterial properties (Verma and Singh, 2008). Plants contain a number of components and secondary metabolites that contribute to their antimicrobial activity, such as alkaloids, tannins, flavonoids, terpenoids, steroids and phenols (Ciocan and Bara, 2007; Gyawali and Ibrahim, 2014; Sibanda and Okoh, 2007). Pal and Shukla (2003) also pointed to the fact that plant constituents have been used to treat liver diseases, heart diseases, depression, pain, asthma, hypertension,

neurological disorders, irritable bowel syndrome, cancer and so on. Additionally, Sibanda and Okoh (2007) suggested that plants have compounds; each one could be effective against pathogenic bacteria, notably antibiotic resistant strains directly, or indirectly by affecting bacterial cell-sensitivity to antibiotics.

1.1.2 Aromatherapy

Aromatherapy employs essential oils, and other aromatic compounds to help achieve better health (Chen *et al.*, 2016).

1.1.2.1 Essential oils

Essential oils (EOs) are secondary metabolites which are obtained from various parts of aromatic plants, such as fruits, leaves, roots, peels, flowers, barks, buds, herbs, wood and seeds (Burt, 2004; Kurdelas *et al*., 2012; Tongnuanchan and Benjakul 2014). They also named volatile odoriferous oils or ethereal oils (Burt, 2004). 'Essential oil' as a term was first used by Paracelsus who, in the 16th century, named the effective component of any medicine *Quinta essential* (Burt, 2004; Edris, 2007). Tongnuanchan and Benjakul (2014) described EOs as a complex mix of volatile compounds in a concentrated liquid form. Several methods are currently used to extract these compounds, including steam distillation, fermentation and expression,

but steam distillation is often employed especially for commercial production (Burt, 2004). Such an approach was used 2000 years ago in the East (Persia, Egypt and India) and modified in the 9th century through the Arabs (Burt, 2004). Burt (2004) commented that differences exist in the constitution of essential oils depending on the geographical location and harvesting seasons and even in material taken from different parts of the same plant. EOs for examples possess the most marked antimicrobial activity when harvested during, or directly after, flowering (Burt, 2004). EOs possess a large range of components, including phenols, carbohydrates, alcohols, aldehydes, ethers and ketones (Sokovic *et al*., 2010), many of which exhibit alternative natural antibacterial properties (Gutierrez *et al*., 2008). Compounds like, terpinene-4-ol, *cis*-ocimene, camphor, 1,8-cineole, linalool, γ -terpinene, eugenol, chamazulen, perillaldehyde, cinnamic acid, thujone, α-bisabolol, carvacrol, thymol, *p*-cymene, cinnamaldehyde, and limonen have been shown to be effective antimicrobial components extracted from essential oils (Seow *et al*., 2014), and the antimicrobial activity of essential oils is generally effective aginst both Gram-positive and Gram-negative bacteria (Burt, 2004; Sokovic *et al*., 2010; Tongnuanchan and Benjakul, 2014); although as a generalization, Gram-negative bacteria are usually the most resistant to the action of

essential oils (Burt, 2004; Seow *et al*., 2014). A number of mechanisms of action and a similar large number of target sites in the bacterial cell have been recognized; such sites include: degradation of the cell wall, damage to the cytoplasmic membrane, leakage of cell contents and coagulation of cytoplasm (Burt, 2004). Furthermore, Mikulášová *et al.* (2016) stated that that the antimicrobial effects of essential oils are based on their hydrophobicity, which enables them to pass into the lipid layer of both the bacterial cell membrane and the mitochondria of eukaryotic cells and there produce disorder of the cytoplasmic membrane thereby causing a rise in membrane permeability, especially to H^+ and K^+, which finally leads to cell lysis and death.

1.1.3 Combination therapy

Combination therapy, based on the use of more than one or more drugs, is widely used and is often beneficial in the war against infections. The use and misuse of antibiotics, over many years, has led to a rise in resistant microorganisms and bacteria are now classified as single or as being multiple antibiotic resistant (Aiyegoro *et al*., 2009; Chanda and Rakholiya, 2011). 90-95% of *Staphylococcus aureus* globally are resistant to penicillin and, in the majority of Asian countries, nearly three quarters of this bacterium are methicillin resistant (Chanda and Rakholiya, 2011). Chanda

and Rakholiya (2011) pointed out that the speed of emergence of the increasing antibiotic resistant bacteria is outpacing the introduction of new antimicrobial agents; while a limited life expectancy exists in new families of antibiotics. A possible alternative strategy is the use of plant extracts, individually and/or in combination with antibiotics (Chanda and Rakholiya, 2011; Van Vuuren *et al*., 2009). Antibiotics on their own often do not have the desired inhibitory effects against pathogens although a combination of antimicrobial agents can result in a synergistic effect (Aiyegoro and Okoh, 2009; Chanda and Rakholiya, 2011). Consequently, combinations of plants are used by traditional healers to treat diseases (Aiyegoro and Okoh, 2009). Antibiotics act differently in the bacteria cell, depending on the antibiotic compounds and their cell targets. For example, penicillin inhibits cell wall biosynthesis, weakening the wall and finally killing the bacteria, while other antibiotics inhibit DNA replication, RNA synthesis and protein synthesis (Chanda and Rakholiya, 2011). Clinically, combinations of two or more antibiotics exhibit a range of different modes of action in order to avoid the development of antibiotic resistance (Aiyegoro *et al*., 2009). However, combinational antibiotic therapy is not effective for long periods because of changes in microbial susceptibility (Aiyegoro *et al*., 2009). *In vitro*, Aiyegoro *et al.* (2009) suggested that some plants have compounds

that can enhance the activity of some β-lactam antibiotics by attacking the same site in the cell wall directly. Alternatively, when antibiotics, for example, chloramphenicol, ciprofloxacin, erythromycin and tetracycline, target other sites in the bacteria cell, some plant-derived compounds can inhibit MDR efflux systems, these being responsible for a major level of bacterial resistance to antibiotics (Aiyegoro *et al*., 2009). Combination therapy brings significant advantages due to the synergistic interactions between either different antibiotics or plant extracts or antibiotic and plant extract, these advantages leading to an increase in treatment efficacy, the treatment of mixed infections, the prevention of resistant strains emerging and a decrease in recovery time (Chanda and Rakholiya, 2011; Van Vuuren *et al*., 2009). Moreover, the combination can minimise any adverse toxic effects and reduce the required dose of drugs (Aiyegoro and Okoh, 2009; Aiyegoro *et al*., 2009). Some plant antimicrobials fail to exhibit any antimicrobial properties but do so when combined with a wide variety of standard drugs (Chanda and Rakholiya, 2011; Aiyegoro and Okoh, 2009); for example, *in vitro*, the antibacterial activities of amoxicillin alone or in combination with plant extracts for *Emblica officinalis* and *Nymphae odorata* against MRSA were reported (Mandal *et al*., 2010). Darwish *et al.* (2002) found that some Jordanian plant materials enhance the effect of

gentamicin and chloramphenicol against *S. aureus* and Adwan *et al.* (2010) reported that combinations of antibiotics with different plant extracts against *Staphylococcus aureus* resulted in synergistic effects; such effects have been seen against Gram- negative bacteria.

1.2 Wound Microbiology and Management

A wound is the physical injury, which causes an opening or breaking in the skin (Alam *et al*., 2011). From a microbiological view, the obvious main function of skin is to control microbes on its surface and to prevent invasion of underlying tissues by potential pathogens (Bowler *et al*., 2001). However, the appropriate way for healing of wounds is necessary for the restoration of disrupted skin function (Alam *et al*., 2011). Wounds provide a warm, moist environment, which encourages microbial colonization and growth especially where foreign material and dirt is present (Bowler *et al*., 2001).

Wounds can be generally regarded as being acute or chronic, with the former being caused by external damage to intact skin and include bites, burns, surgical wounds, abrasions and minor cuts. Simple wounds generally heal within a short period without any medical intervention. More severe traumatic injury, on the other hand, such as burns often need to be debrided and treated with antimicrobials (Bowler *et al*., 2001). Currently, about

6,000,000 of people suffer from chronic wounds globally (Alam *et al*., 2011).

Over the years, plants and plant extracts have been considered as folklore medicine in treatment for cuts, wounds, and burns as they have wound healing properties that could be developed into phytotherapeutic agents in order to treat and manage wounds and their associated complications (Ashoka Babu *et al*., 2012). Ashoka Babu *et al.* (2012) stated that plants and their extracts need to be recognized for the treatment and management of wounds and for this purpose and many studies have been carried out in recent times.

1.3 Some important pathogenic microorganisms

1.3.1 *Staphylococcus aureus*

Staphylococcus aureus is a common Gram-positive, coccal skin organism which microscopically appears as grape-like clusters. It is a facultative anaerobe causing skin and soft tissue infections, meningitis, osteomyelitis, food poisoning, pneumonia, endocarditis and toxic shock syndrome (Ghalem and Mohamed, 2008). This bacterium produces several enzymes such as coagulase and catalase (Ghalem and Mohamed, 2008). In the hospital environment and long term care facilities, *Staphylococcus*

aureus is often highly resistant to antibiotics (Chanda and Rakholiya, 2011).

1.3.1.1 Methicillin-resistant *Staphylococcus aureus* (MRSA)

MRSA is a resistant organism which usually causes skin infections; it is resistant to beta-lactam antibiotics, including penicillin, methicillin, oxacillin and amoxicillin, and is responsible for many resilient infections. Levy and Marshall (2004) reported that rare cases of penicillin-resistant *Staphylococcus aureus* appeared in London as early as the late 1940s. Respectively, in 1961, methicillin resistance appeared, followed by vancomycin resistance in 1968 and zyvox in 1999 (Palumbi, 2001). In the last decade, linezolid, daptomycin, telavancin and ceftaroline are medicines that are approved to cure infections caused by drug-resistant Gram-positive pathogens; although they have significant limitations (Choo and Chambers, 2016). There are two types of MRSA in existence, namely community-acquired MRSA (caMRSA) and hospital acquired MRSA (haMRSA). Initially, all reported MRSA infections were HA-MRSA, although in 1982, resistant strains spread outside hospitals into the community (CA-MRSA), notably for example, in Detroit, among intravenous drug users (Chao *et al*., 2008). Resistance to β-lactam antibiotics occurs at the bacterial cell wall, with penicillin-binding proteins, which induce methicillin resistance in *S.*

aureus (MRSA). These bacteria possess a genetic element called the Staphylococcal cassette chromosome mec (SCCmec) a *mecA* gene, which codes responsible for the creation of an altered penicillin-binding protein (PBP2a) (Mulvey and Simor, 2009; Zinner, 2007).

1.3.3 *Escherichia coli*

Escherichia coli is a rod-shaped Gram-negative, facultative anaerobic bacterium, motile with an optimum growth at 37°C and pH between 6.4 and 7.2 (Manderson *et al*., 2006). The intestines of animals and humans are the usual environments for *E. coli,* which is responsible for many lower urinary tract infections (Ghalem and Mohamed, 2008; Masters *et al*., 2011; Welch, 2006), including urethritis and cystitis as well as wound and gallbladder and bile duct infections, meningitis and peritonitis (Ahmed *et al*., 2007; Masters *et al*., 2011; Welch, 2006); it is a frequent bacterial infection in the urinary tract, causing 150 million cases globally per year (Soubirou *et al*., 2015).

1.3.4 *Candida rugosa*

Candida rugosa appears as wrinkled colonies, white to cream, with a diameter of approximately 5 μm, and under a microscope, branched pseudohyphae are seen with chains of elongated blastoconidia (Padovan *et al*., 2013). It is found in the environment and also causes bovine mastitis

diseases (Padovan *et al*., 2013). This yeast has recently been identified as an emerging fungal pathogen (Pfaller *et al*., 2006; Tay *et al*., 2011), since it causes invasive infection in immune-compromised patients (Tay *et al*., 2011), particularly in patients undergoing prolonged antibiotic treatment. However, *C. rugosa* is recognized as causing candidemia in trauma patients (Padovan *et al*., 2013). Importantly, *C. rugosa* is exhibiting declined susceptibility to amphotericin B and fluconazole (Padovan *et al*., 2013; Pfaller *et al*., 2006; Tay *et al*., 2011).

1.3.5 *Candida inconspicua*

Candida inconspicua appears as a budding, oval yeast with elongated blastoconidia (D'Antonio *et al*., 1998); it is similar in morphology to *Candida krusei* (Essayag *et al*., 1996) and, additionally, it is difficult to differentiate *Candida inconspicua* from *Candida norvegensis* by using traditional methods (Guitard *et al*., 2015). Meurman *et al.* (2007) reported that the fourth most common bloodstream infection associated with hospitals in the U.S. is caused by *Candida* and frequently *Candida inconspicua* is isolated from invasive infections in immune-compromised hosts; thus it is considered as an emerging fungal pathogen (Guitard *et al*., 2015, Loeffler *et al*., 2000) which is exhibiting fluconazole-resistant emerging species (Guitard *et al*., 2015; Majoros *et al*., 2005).

1.4 Bacterial resistance to antibiotics

Antibiotic-resistant pathogens are a major world health problem, particularly in hospitals and other health care facilities. Mulvey and Simor (2009) reported that bacteria can be resistant because:

1. Chemically, they can alter antibiotic targets by enzymatic inactivation.

2. Physically, bacteria can change the drug's structure.

3. They disable potential antibiotic-target sites.

Antibiotic resistance in bacteria can be either intrinsic or acquired. Acquired resistance is a kind of bacterial resistance that gives bacteria new genetic material via a mutation (only one gene mutation per $10^{7} - 10^{10}$ bacteria) or by the acquisition of a new plasmid or transposon (Mulvey and Simor, 2009). Natural (or intrinsic) resistance to antibiotics is mediated by structures like capsules or results from membrane permeability (Mulvey and Simor, 2009). Generally Gram-negative bacteria are more resistant to antibiotics than are Gram-positive bacteria (Nikaido, 1998); because they have complex cell walls as well as permeability barriers 'porins' in their outer membranes; such obstacles act as barriers to prevent the access of

toxic compounds, for example antibiotics, to targets within the bacterial cell (Miller, 2016). Carlet *et al.* (2012) showed that antibiotic resistance in hospitals and communities is increasingly linked to the misuse or overuse of antibiotics by patients and/or physicians. The same authors stated that the loss of antibiotic effectiveness against different infections is due to a) antibiotics are prescribed for infections caused by viruses e.g. colds and influenza, b) doctors in some cases advise their patients to take antibiotics just to be safe or to avoid the occurrence of secondary bacterial infections, c) patients fail to complete a full course of treatment, d) patients often pressurize doctors to prescribe antibiotics. All such examples of misuse or overuse can contribute to an increase in the problem of bacterial resistance (Yap *et al.,* 2014). In conclusion, a large number of infections result from infection by bacteria such as *Staphylococcus aureus* which are resistant to both penicillin and to methicillin (Palumbi, 2001).

1.5 A world without antibiotics and the need to find alternatives

The use of antibiotics is creating a number of problems including:

1. Breast cancer can be caused as a result of prolonged antibiotic use (Velicer *et al.,* 2004).

2. Antibiotic effects on normal flora; Francino and Moya (2013) reported

that the mutualistic communities of microorganisms existing as the normal flora on or in the human body can be altered by the use of antibiotics.

3. The prolonged use of antibiotics can lead to immune suppression (Vanvlem *et al*., 1996).

4. Antibiotics can cause pollution; the transport of the waste of human and veterinary antibiotics into soil can cause important changes in the diversity of soil microorganisms, e.g. the bacterial population may be reduced, while the fungal population can be increased (Mojica and Aga, 2011).

1.6 The main impacts of antibiotic resistance

The increase of antibiotic resistance is significantly associated with:

1. The cost of treatment for antimicrobial resistance, which has increased dramatically due to the development of bacterial resistance and as a result, has become an economic burden. For example, in Canada, the cost of treating MRSA infections in hospital was $14,360 per patient in 2001 (Mulvey and Simor, 2009).

2. An increase in morbidity and mortality rates in nosocomial infection; Levy and Marshall (2004) reported that the rate of antibiotic-resistant infections has increased mortality and morbidity in comparison with drug-

susceptible infections; in New York City, for example, the rate of mortality has increased three-fold from 8% to 21% and the cost has increased by 22% because of treatment of methicillin-resistant (MRSA) instead of methicillin-susceptible (MSSA) *S. aureus*.

1.7 Research objectives

The overall aim of this study was to find new and effective natural plant products as alternatives to antibiotics. The first part of the study is explored to a broad screening programme aimed at finding the most effective antibacterial herbs and their extracts, with a view to their further study.

Chapter Two: Antimicrobial Activity of Herbal Extracts

2.1 Introduction

2.1.1 Alternative herbal antibiotics

The following plant-derived products have been shown to have antibiotic properties:

2.1.1.1 Derum

Juglans regia bark (called Derum) is obtained from the walnut tree and is usually used by women to colour their lips and clean their teeth in India, Pakistan and the Middle East (Ashri and Gazi, 1990; Darmani *et al*., 2006); recently, it has been shown to have antimicrobial activity against a number of pathogenic microorganisms (Zakavi *et al*., 2013). The main active ingredients are resins, glycosides, juglonic acid, volatile oil, tannic acid and phenolic acid (Darmani *et al*., 2006); the bark of the walnut is also used for its anti-inflammatory and anticancer properties and as a laxative, and diuretic (Zakavi *et al*., 2013).

2.1.1.2 Grapefruit seed extract (GSE)

Grapefruit seed extract (*Citrus paradisi*) is removed from the pulp and seeds of grapefruit grown in Morocco, Spain, South Africa, Jordan, Brazil, Jamaica, Asia and Mexico (Gupta *et al*., 2011). According to these authors, GSE is used around the world as a traditional medicine due to it being:

antimicrobial, antibacterial, anti-inflammatory, antifungal, antiviral, a preservative and an antioxidant. Additionally, GSE has been used to treat cancer, for cellular regeneration and heart-health maintenance, for the lowering of cholesterol and the treatment of lupus nephritis and rheumatoid arthritis (Gupta *et al*., 2011). It is used in detergents, soaps, cosmetics and perfumes (Gupta *et al*., 2011) and contains flavonoids, for example naringin and limonoid, kaempferol, quercetin, as well as citric acid compounds, which give the extract its antimicrobial activity (Cvetnic and Vladimir-Knezˇevic´ 2004; Jang *et al*., 2011; Choi *et al*., 2014).

2.1.1.3 Noni juice

Morinda citrifolia is a traditional remedial plant, which it's common name ranging from Indian mulberry, Bajitian in China, Nono in Tahiti, and noni in Hawaii (Ahmad *et al*., 2012). For over 2000 years, Polynesians have used it to treat various diseases and to stimulate the immune system (Ahmad *et al*., 2012). The bark and the roots of Noni have also been used as a pigment for dyeing clothes (Palu *et al*., 2008). Chan-Blanco *et al.* (2006) reported that Noni contains some 160 compounds, the most important compounds being: phenols like anthraquinones (morindone, damnacanthal, and morindin.), and also, asperuloside, scopoletin and aucubin; caprylic acids and caproic; alkaloid-xeronine and amino acids, such as isoleucine,

aspartic acid and glutamic acid. The above authors mentioned that Noni plants inhibit the growth of bacteria such as: *Proteus morgaii, Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Helicobacter pylori, Escherichia coli, Shigella* and *Salmonella* due to phenolic compounds such as scopoletin.

2.1.1.4 Goldenseal

Goldenseal (*Hydrastis canadensis*) is a perennial plant known as yellowroot, orangeroot, eyebalm and eyeroot. Native Americans used the roots of Goldenseal herbs in remedies for a number of illnesses such as skin and eye infections, inflammation, like a tonic to increase appetite and to finally stimulate digestion (Weber *et al*., 2003). The commercial product of Goldenseal is available as tablets and capsules, liquid extracts, and glycerides (low alcohol extracts). Goldenseal contains five alkaloids, namely: hydrastinine, palmatine, hydrastine, canadine and berberine. The most important is berberine (Figure 2.1) because it has antibacterial activity and is antiseptic, anti-diabetic, an astringent, anti-inflammatory, laxative and a bitter tonic (Chen *et al*., 2013).

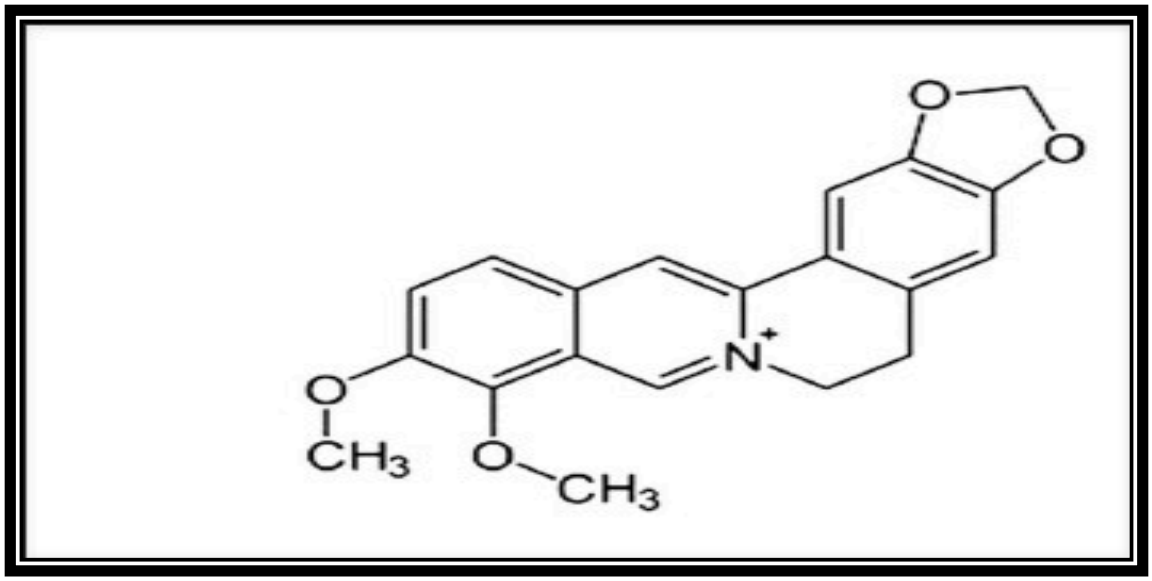

Figure 2.1 The chemical structure of berberine (Yang *et al.,* 2011).

The aim of the experimental work described in this chapter was to explore a broad screening programme aimed at finding the most effective antibacterial herbs and their extracts.

2.2 Materials and methods

2.2.1 Plant products samples

The following herbal products were used: Antioxidant detox cleanse, Glyco-x 500, Broccoli sprout extract, *Gracinia cambogia*, Grapefruit seed extract (GES), Goldenseal liquid (DL) and capsule (D), Noni juice (N) and Derum (Dr). Derum was obtained from local shops in Saudi Arabia and all other plant extract samples were obtained commercially from Amazon; and all samples were stored at room temperature until used.

2.2.2 Plant product sample preparation

2.2.2.1 Derum preparation

Bark of *Juglans regia* was cut into small pieces and then ground to a powder with a mill. Aqueous extracts were prepared by mixing 15 g of the powder with 100 ml of sterile distilled water and shaking for 48 hours at 4℃. The mixture was then homogenized in a household blender for one minute at full speed and the homogenate was filtered through a double layer of cheesecloth and centrifuged at 3000xg for 10 minutes. The supernatant was finally filtered through a sterile membrane filter (0.45 μm) and stored at -20℃ for later use; this supernatant is called aqueous extract (Darmani *et al*., 2006; Janakat *et al*., 2004).

2.2.2.2 Goldenseal capsule (D) preparation

Goldenseal capsules were opened to collect the powder inside. Each capsule contained 400mg of Goldenseal leaf and flower extract; aqueous extracts were prepared by mixing 400 mg of the powder with 10 ml of sterile distilled water. Others plant product samples were used directly as purchased; various sample concentrations from 5% to 100% (v/v) were prepared by dissolving samples with sterile distilled water.

2.2.3 Test organisms

The following test microorganisms were used: *Staphylococcus aureus*, Methicillin-Resistant *S. aureus* (MRSA strain USA300)*, Escherichia coli*, *Candida rugosa* and *Candida inconspicua*.

2.2.4 Inoculum preparation and turbidity standard

The inocula of susceptibility tests were adjusted to $1.5x10^8$ CFU/ml which reference to the (0.5 McFarland standards). Two to five isolated colonies from pure cultures were taken with a sterile loop, and inoculated into a tube containing distilled sterilized water and mixed using a vortex until a homogenate was formed. The turbidity was measured by spectrophotometer at 600 nm for bacteria, and at 580 nm for yeast. These suspensions were used within 30 minutes of preparation (Andrews, 2001).

2.2.5 Determination of microbial contamination of plant products

In order to identify and detect any bacteria or fungi contaminating the plant products chosen in this study, isolation of microorganisms from samples was carried out using Nutrient agar and Sabouraud Dextrose agar (incubated at 37°C under aerobic conditions for 24hrs). Sabouraud Dextrose agar plates were incubated at 28°C under aerobic conditions for 48hrs.

2.2.6 Agar diffusion assay

The plates were prepared using 20 ml of sterile media. Bacterial or yeast suspension (0.1ml) was poured into each plate containing Muller-Hinton agar for bacteria or Sabouraud Dextrose agar for yeasts. A sterile glass spreader was used to distribute the inoculum across medium surface. All plates were allowed to dry for 30 minutes. Wells (8.0 mm in diameter), were cut from the culture media using a sterile metal cylinder, and then filled with 0.1 ml; the test samples had concentrations of 100%, 80%, 50%, 25% and 5% v/v. After a 30 min pre-diffusion time interval, the Petri dishes were incubated at 37 °C for 24 h for bacteria and at 28°C for 48h for yeasts and any inhibition zone around the well was measured in mm (the values given include the well diameter (8.0mm)). The result was then recorded. All experiments were carried out in triplicate. The effectiveness of antimicrobial plant extracts was determined using the well diffusion assay.

2.2.7 Determination of the minimum inhibitory concentration (MIC)

The minimal inhibitory concentration (MIC) was determined by the macro broth dilution assay method. Each tube-contained 2ml Mueller-Hinton broth medium for bacteria or Saboraud Dextrose broth for yeasts with sample concentrations ranging from 0.8 % to 100% v/v. Each tube received 0.02 ml of bacterial or yeasts suspension adjusted to 0.5 McFarland turbidity standard. Controls without plant extracts, without bacterial or yeasts inoculum or with plant extracts only were also included. The mixtures were homogenized using a Vortex mixer for 2 minutes, and the tubes were then incubated at 37°C with shaking at 250 rpm for 24 hours for bacteria and 48 hours in 28°C with shaking at 180 rpm for yeasts. The first tube in the above series with no visible growth was marked as the MIC.

2.2.8 Determination of the minimum bactericidal concentration

Bacterial culture (0.01ml) was taken from the tubes with no growth and plated onto Mueller–Hinton agar, and then incubated overnight at 37°C to determine MBC. The lowest concentration, which showed no growth on the agar, was defined as the MBC.

2.2.9 Determination of the minimum fungicidal concentration

After determining the MICs, 0.01ml was taken from the tubes with no

growth and plated onto Saboraud Dextrose agar, and then incubated at 28°C; MFCs were recorded after 48 hours. The MFC was defined as the lowest concentration that resulted in no growth in these plates.

2.2.10 Determination of the effect of the plant products after mixing with honey

The plates were prepared using 20 ml of sterile media. A total of 0.1 ml of bacterial and yeast suspension were poured on to each plate containing Muller-Hinton agar for bacteria or Sabouraud Dextrose agar for yeasts. A sterile glass spreader was used to distribute the inoculum on the surface of agar. All plates were allowed to dry for 30 minutes. Wells, 8.0 mm in diameter, were cut from the culture media using a sterile metal cylinder, and then filled with (0.05 ml 24+ Manuka honey +0.05 ml of the test samples). Each well received 0.1ml of mixture. After a 30 min pre-diffusion time interval, the Petri dishes were subsequently incubated at 37 °C for 24 h for bacteria and at 28°C for 48hrs for yeasts and the inhibition zone around each well was measured in mm, including the well (8.0mm). The result was then recorded. Plant extracts alone (0.1ml of 50% v/v) were used as the control.

2.2.11 The antibacterial activity of herbal extracts after being autoclaved

Plant products were autoclaved at 120°C for 15 minutes, and thier antibacterial activity was then measured against all tested bacteria using the agar well diffusion method. Non- autoclaved samples of plant products acted as the control.

2.2.12 Statistical Analysis

All observations were presented as Mean ± SD (Standard Deviation). The data were analysed by IBM Corp© 24.0. One way ANOVA was performed to compare if there was a significance difference of the inhibition zone values measured between the different extracts against the test organisms. $P \leq 0.05$ was considered as statistically significant. Tukey-Post-Hoc test confirmed the pairwise comparisons.

2.3 Results

2.3.1 Determination of microbial contamination of plant products

No microbial contamination was found in any of the samples of plant products.

2.3.2 Agar diffusion assay

In this study, nine plant products were tested at different concentrations, i.e. 100%, 80%, 50%, 25% and 5%v/v for their antimicrobial activity against Gram-negative bacteria and Gram-positive bacteria including methicillin-resistant *S. aureus* (MRSA) and two yeast species. Only five of the plant products showed antimicrobial activity against at least two of the microorganisms tested using the agar diffusion assay (Tables 2.1-2.5); Goldenseal (DL) showed the most potent activity against almost all of the microorganisms studied (Tables 2.1, 2.2, 2.4 and 2.5) and there was a statistically significant difference between Goldenseal (DL) and others herb extracts ($P=0.001$). *Staphylococcus aureus* and MRSA were the most susceptible to all plant products tested (Tables 2.1).

In the case of *E.coli*; Goldenseal, either as a liquid (DL) or capsule (D) had no effect even at the highest concentration (100%) (Table2.3) ($P>0.05$). In addition, Grapefruit seed extract (GSE) and Goldenseal capsule (D) failed

to form inhibition zones against any of the yeasts used (Tables 2.4, 2.5). Noni juice did, however, produce an inhibition zone against *Candida rugosa*, but not *Candida inconspicua* (Tables 2.4, 2.5).

An increase in the concentration of the plant products generally produced greater inhibition as shown (Figs. 2.2-2.4) by the diameter of zone of inhibition, showing that the zones of inhibition resulted from the bactericidal or bacteriostatic action of plant products on the tested organisms.

Table 2.1 The effect of various concentrations of plant extracts against *S. aureus* determined by the agar diffusion assay. Means of triplicates (diameter mm including well (8.0 mm)) ± Standard Deviation.

	Plant Product concentrations				
Type of Plant Products	**100%**	**80%**	**50%**	**25%**	**5%**
Grapefruit seed extract	21.3±0.5	18.6±0.5	14.3±1.1	8±0	8±0
Goldenseal (D)	12.3±0.5	11.3±0.5	10.6±0.5	8±0	8±0
Goldenseal (DL)*	25±0	23.1±0.2	22.5±0.5	18.6±0.5	13.3±0.5
Noni juice	11.6±0.5	8±0	8±0	8±0	8±0
Derum	21.6±0.5	20±0	16.6±0.5	11.6±0.5	8±0
Antioxidant detox cleanse	8±0	8±0	8±0	8±0	8±0
Glyco-x 500	8±0	8±0	8±0	8±0	8±0
Broccoli sprout extract	8±0	8±0	8±0	8±0	8±0
Gracinia cambogia	8±0	8±0	8±0	8±0	8±0

(*Statistically significant differences at $P \leq 0.05$)

Table2.2 The effect of various concentrations of plant extracts against MRSA determined by the agar diffusion assay. Means of triplicates (diameter mm including well (8.0 mm)) ± Standard Deviation.

	Plant Product concentrations				
Type of Plant Products	**100%**	**80%**	**50%**	**25%**	**5%**
Grapefruit seed extract*	17±0	16.1±0.2	16±0	8±0	8±0
Goldenseal (D)*	10.3±0.5	8±0	8±0	8±0	8±0
Goldenseal (DL)*	23.3±0.5	21±1	19.6±0.5	18.3±0.5	14.3±0.5
Noni juice*	14±1	8±0	8±0	8±0	8±0
Derum*	20±0	17.8±0.2	15.3±0.2	13.6±0.5	8±0
Antioxidant detox cleanse	8±0	8±0	8±0	8±0	8±0
Glyco-x 500	8±0	8±0	8±0	8±0	8±0
Broccoli sprout extract	8±0	8±0	8±0	8±0	8±0
Gracinia cambogia	8±0	8±0	8±0	8±0	8±0

(*Statistically significant differences at $P \leq 0.05$)

Table2.3 The effect of various concentrations of plant extracts against ***E.coli*** determined by the agar diffusion assay. Means of triplicates (diameter mm including well (8.0 mm)) ± Standard Deviation.

	Plant Product concentrations				
Type of Plant Products	**100%**	**80%**	**50%**	**25%**	**5%**
Grapefruit seed extract*	17.8±0.7	14±0	8±0	8±0	8±0
Goldenseal (D)	8±0	8±0	8±0	8±0	8±0
Goldenseal (DL)	8±0	8±0	8±0	8±0	8±0
Noni juice*	10.6±0.5	8±0	8±0	8±0	8±0
Derum*	12.3±0.5	11.3±0.5	11±0	9.3±1.1	8±0
Antioxidant detox cleanse	8±0	8±0	8±0	8±0	8±0
Glyco-x 500	8±0	8±0	8±0	8±0	8±0
Broccoli sprout extract	8±0	8±0	8±0	8±0	8±0
Gracinia cambogia	8±0	8±0	8±0	8±0	8±0

(*Statistically significant differences at $P \leq 0.05$)

Table2.4 The effect of various concentrations of plant extracts against ***Candida rugosa*** determined by the agar diffusion assay. Means of triplicates (diameter mm including well (8.0 mm)) ± Standard Deviation.

	Plant Product concentrations				
Type of Plant Products	**100%**	**80%**	**50%**	**25%**	**5%**
Grapefruit seed extract	8±0	8±0	8±0	8±0	8±0
Goldenseal (D)	8±0	8±0	8±0	8±0	8±0
Goldenseal (DL)*	18±1	16.6±1.1	15.3±2	8±0	8±0
Noni juice*	12.3±0.5	8±0	8±0	8±0	8±0
Derum*	11±0	8±0	8±0	8±0	8±0
Antioxidant detox cleanse	8±0	8±0	8±0	8±0	8±0
Glyco-x 500	8±0	8±0	8±0	8±0	8±0
Broccoli sprout extract	8±0	8±0	8±0	8±0	8±0
Gracinia cambogia	8±0	8±0	8±0	8±0	8±0

(*Statistically significant differences at $P \leq 0.05$)

Table2.5 The effect of various concentrations of plant extracts against ***Candida inconspicua*** determined by the agar diffusion assay. Means of triplicates (diameter mm including well (8.0 mm)) ± Standard Deviation.

	Plant Product concentrations				
Type of Essential Oils	**100%**	**80%**	**50%**	**25%**	**5%**
Grapefruit seed extract	8±0	8±0	8±0	8±0	8±0
Goldenseal (D)	8±0	8±0	8±0	8±0	8±0
Goldenseal (DL)*	19±1	18±1	14.3±0.5	8±0	8±0
Noni juice	8±0	8±0	8±0	8±0	8±0
Derum*	12.3±0.5	11±0	10±0	8±0	8±0
Antioxidant detox cleanse	8±0	8±0	8±0	8±0	8±0
Glyco-x 500	8±0	8±0	8±0	8±0	8±0
Broccoli sprout extract	8±0	8±0	8±0	8±0	8±0
Gracinia cambogia	8±0	8±0	8±0	8±0	8±0

(*Statistically significant differences at $P \leq 0.05$)

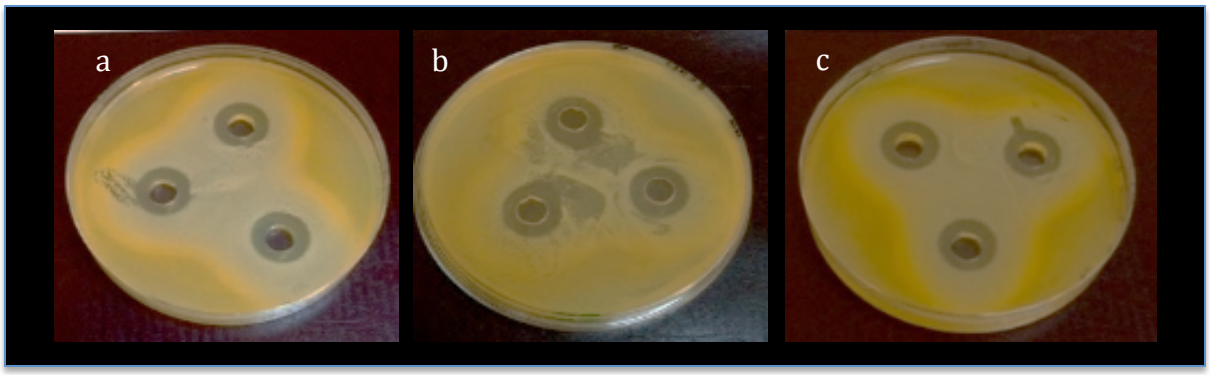

Figure 2.2 Agar diffusion assay for Grapefruit seed extract at various concentrations: 100%, 80 % and 50%. Plates seeded with **MRSA**: (a) zone of inhibition caused by 100 %, (b) zone of inhibition caused by 80% (c) zone of inhibition caused by 50%.

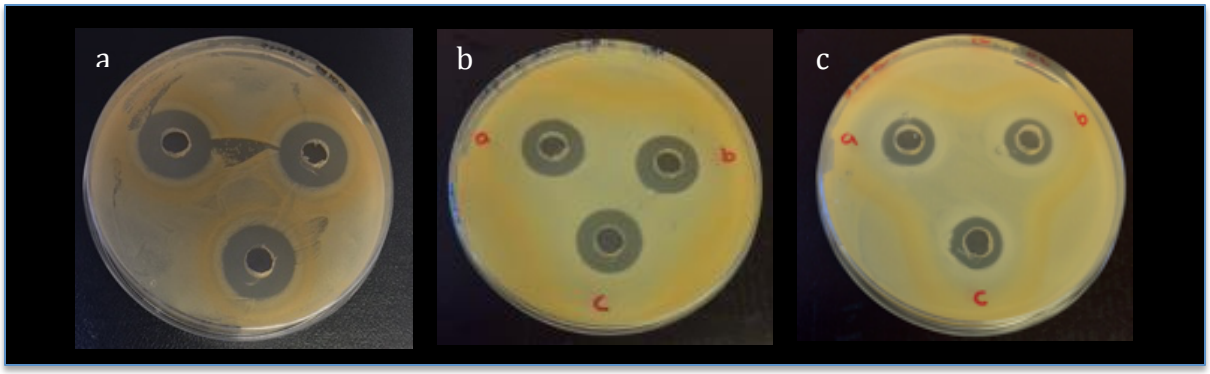

Figure 2.3 Agar diffusion assay for Grapefruit seed extract at various concentrations: 100%, 80 % and 50%. Plates seeded with ***S. aureus***: (a) zone of inhibition caused by 100 %, (b) zone of inhibition caused by 80% (c) zone of inhibition caused by 50%.

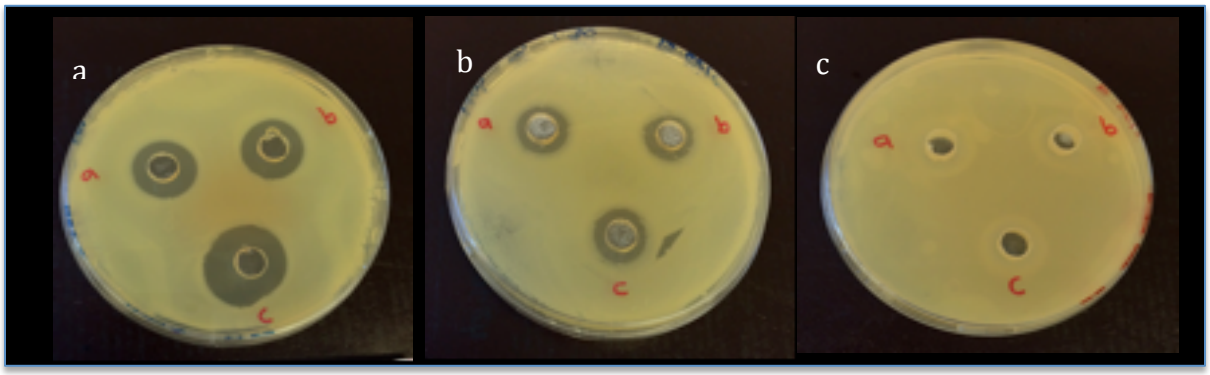

Figure 2.4 Agar diffusion assay for Grapefruit seed extract at various concentrations: 100%, 80 % and 50%. Plates seeded with ***E.coli***: (a) zone of inhibition caused by 100 %, (b) zone of inhibition caused by 80% (c) zone of inhibition caused by 50%.

2.3.3 Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MICs and the MBCs values of all plant products for *E.coli, S. aureus* and methicillin-resistant *S. aureus* (MRSA) are shown in Figs. 2.5-2.7. It was obvious that the Goldenseal (DL) showed the lowest MIC value against MRSA (Fig. 2.7). Grapefruit seed extract effectively inhibited the growth of *S. aureus* and *E.coli* at lowest MIC values as shown in Figures 2.5, 2.6. In general, Derum demonstrated the strongest bactericidal activity against MRSA (12.5%v/v) (Fig.2.7). Derum exhibited the strongest bactericidal activity with *S. aureus* (Fig.2.6), while Grapefruit seed extract recorded the strongest bactericidal activity with *E.coli* (Fig.2.5). Goldenseal (D) was the least active of all of the active plant products and not surprisingly showed the largest MICs and MBCs (Figs. 2.8, 2.9).

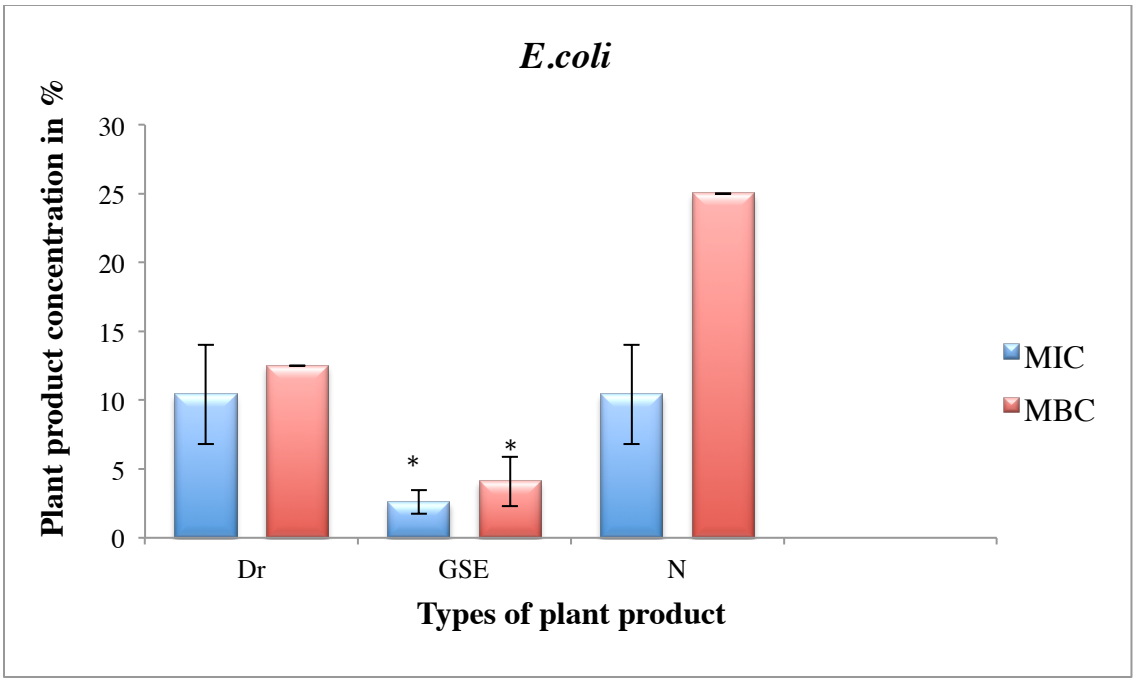

Figure 2.5 Minimum inhibitory concentration (MIC) (%v/v), determined by the macro broth dilution method, and minimum bactericidal concentration (MBC) of different plant products against *E.coli.* The values are means of triplicates ± Standard Deviation.

*Statistically significant differences at P≤0.05.

Grapefruit seed extract= (GSE)

Noni juice= (N)

Derum= (Dr)

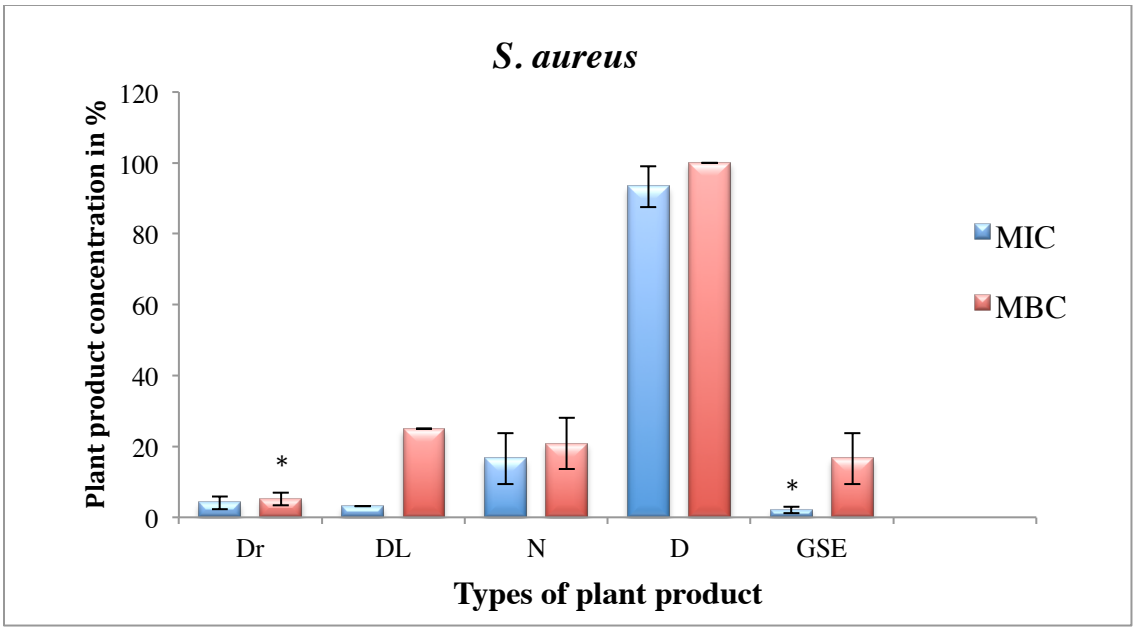

Figure 2.6 Minimum inhibitory concentration (MIC) (%v/v), determined by the macro broth dilution method, and minimum bactericidal concentration (MBC) of different plant products against *S. aureus.* The values are means of triplicates ± Standard Deviation.

*Statistically significant differences at P≤0.05.

There was not a statistically significant difference between GSE and Dr, GSE and Dl in MIC group and Dr and GSE in MBC group (P≥0.05).

Grapefruit seed extract= (GSE)

Goldenseal extract= (DL)

Noni juice= (N)

Goldenseal extract= (D)

Derum= (Dr)

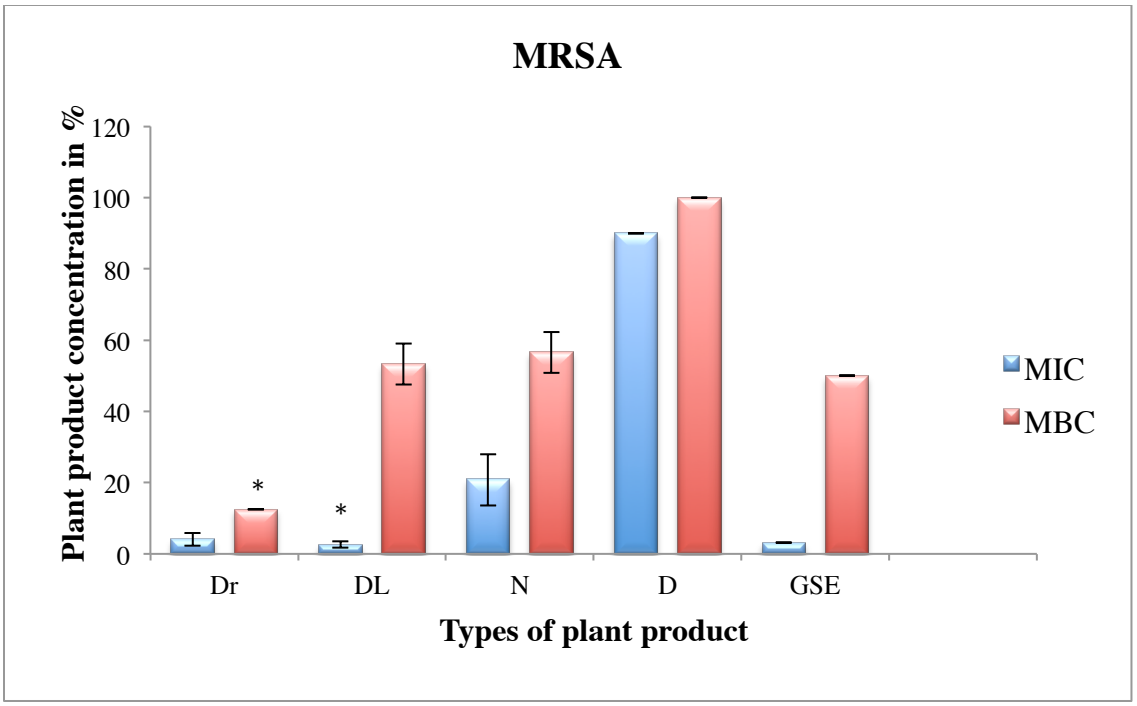

Figure 2.7 Minimum inhibitory concentration (MIC) (%v/v), determined by the macro broth dilution method, and minimum bactericidal concentration (MBC) of different plant products against MRSA. The values are means of triplicates ± Standard Deviation.

*Statistically significant differences at $P \leq 0.05$.

There was not a statistically significant difference between DL and Dr, DL and GSE in MIC group ($P \geq 0.05$).

Grapefruit seed extract= (GSE)

Goldenseal extract= (DL)

Noni juice= (N)

Goldenseal extract= (D)

Derum= (Dr)

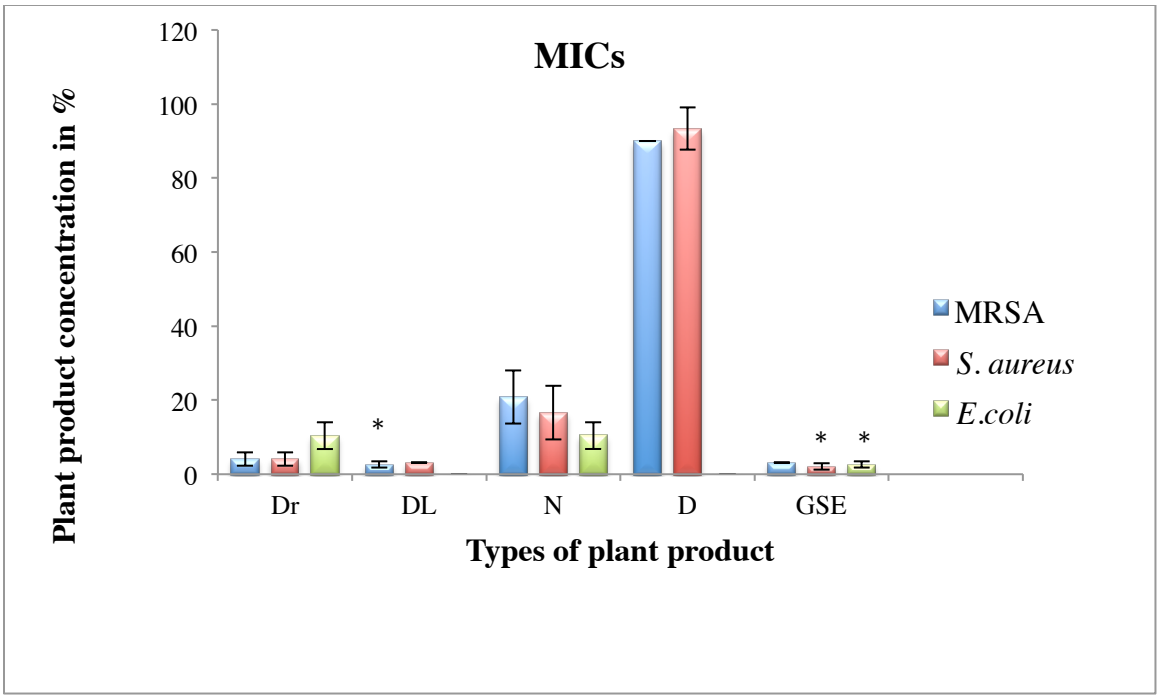

Figure 2.8 The MICs (%v/v) of plant products against pathogenic bacteria. The values are means of triplicates ± Standard Deviation.

*Statistically significant differences at P≤0.05.

There was not a statistically significant difference between DL and Dr, and DL and GSE in MRSA group. There was not a statistically significant difference between GSE and Dr, and GSE and Dl in *S. aureus* group (P≥0.05).

Grapefruit seed extract= (GSE)

Goldenseal extract= (DL)

Noni juice= (N)

Goldenseal extract= (D)

Derum= (Dr)

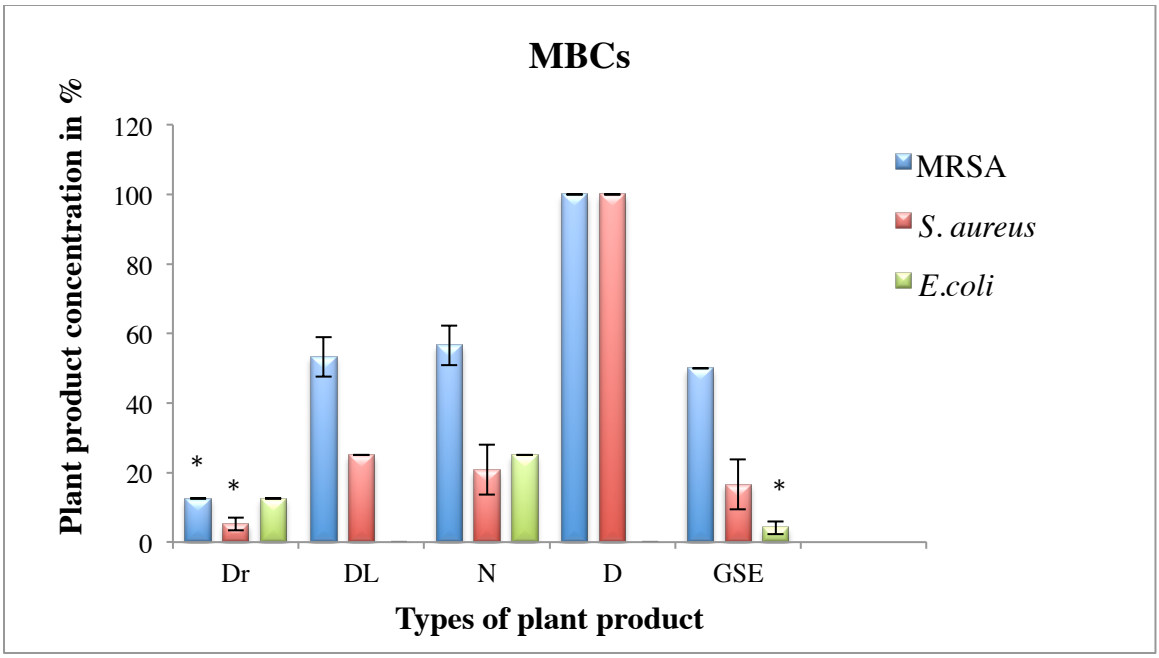

Figure 2.9 The MBCs of plant products against pathogenic bacteria. The values are means of triplicates ± Standard Deviation.

*Statistically significant differences at P≤0.05.

There was not a statistically significant difference between Dr and GSE in *S. aureus* group (P≥0.05).

*Statistically significant differences at P≤0.05.

Grapefruit seed extract= (GSE)

Goldenseal extract= (DL)

Noni juice= (N)

Goldenseal extract= (D)

Derum= (Dr)

2.3.4 Determination of the minimum inhibitory concentration (MIC) and minimum fungicidal concentrations (MFC)

The MIC and MFC values of Goldenseal (DL) and Derum were determined against two types of yeasts (*Candida inconspicua, Candida rugosa*). These MIC values are shown in Figure 2.10 and the MFC values shown in Figure 2.11. Generally, MFC values were higher than MIC values in all results. Goldenseal (DL) effectively had lower MIC values against *Candida rugosa* and also recorded a more marked fungicidal effect than Derum; as did with *Candida inconspicua.*

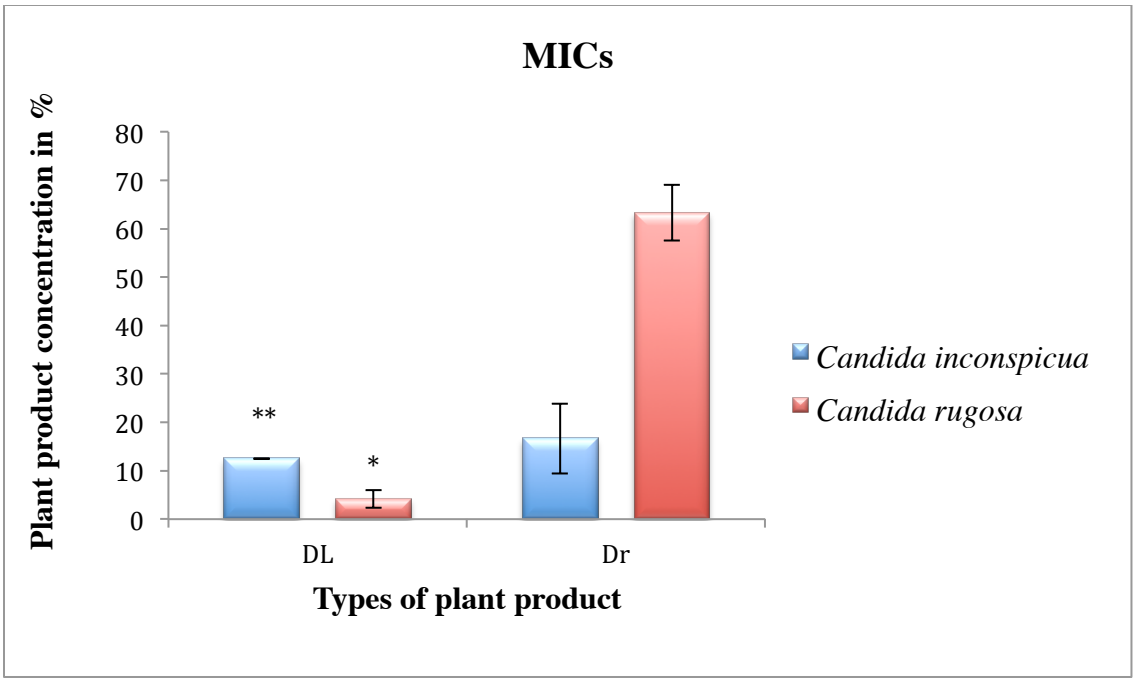

Figure 2.10 The minimum inhibitory concentrations (MICs)(%v/v) of plant products against pathogenic yeasts. Determined by the macro broth dilution method. The values are means of triplicates ± Standard Deviation. *Statistically significant differences at P≤0.05.

** No statistically significant differences at P≥0.05.

Goldenseal extract= (DL)

Derum= (Dr)

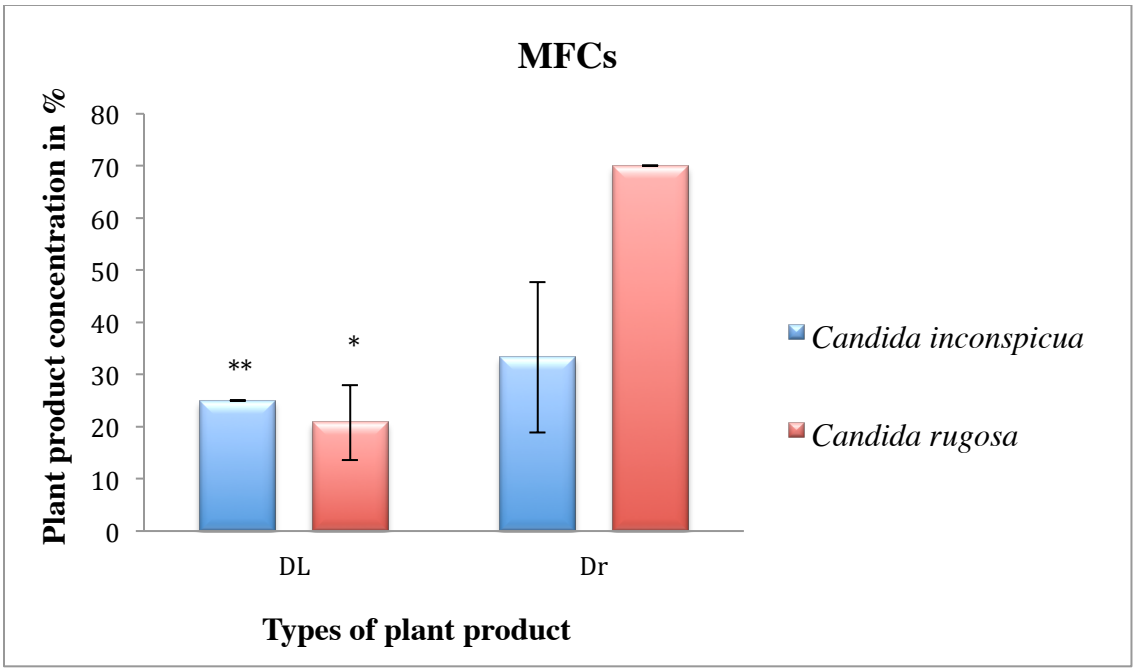

Figure 2.11 The minimum fungicidal concentrations (MFCs) of plant products against pathogenic yeasts. The values are means of triplicates ± Standard Deviation.

*Statistically significant differences at P≤0.05.

** No statistically significant differences at P≥0.05.

Goldenseal extract= (DL)

Derum= (Dr)

2.3.5 Determination of the effect of the plant products after mixing with honey

Different antibacterial and antifungal effects of mixing 24+ Manuka honey with plant products were seen (Table 2.6). Generally, the inhibitory effect after mixing was decreased, or had the same effect, in comparison with each individual plant product. However, Noni juice, Grapefruit seed extract (Figure 2.12) and Goldenseal (D) showed an increase in diameter of the inhibition zone after mixing with Manuka honey 24+ against *S. aureus,* as did Derum with *E. coli* and Noni juice with MRSA (Table 2.6). Interestingly, Goldenseal (DL) presented the same effect after and before mixing with honey against MRSA and *Candida inconspicua* (Table 2.6).

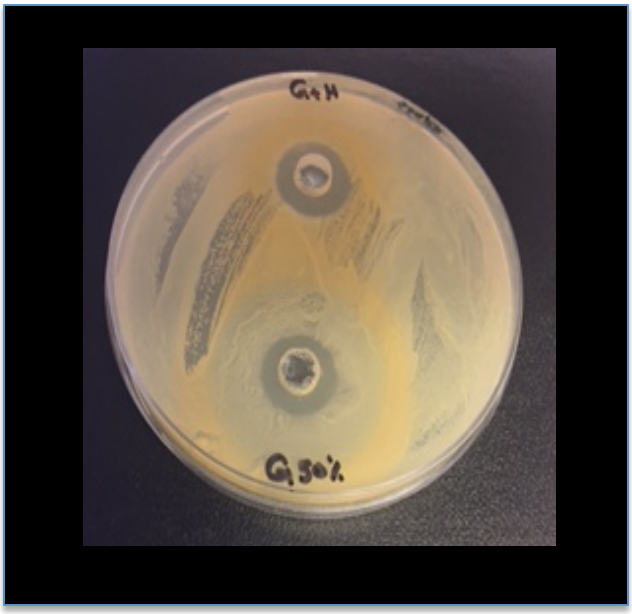

Figure 2.12 Comparison of inhibitions zone for Grapefruit seed extract before and after mixing with Manuka honey 24+; Plate seeded with *S. aureus*.

Table 2.6 Susceptibility pattern of the mixture against different microorganisms determined by well agar diffusion. Means of triplicates (diameter mm including well (8.0 mm)) ± Standard Deviation.

Microbial Strains	**Grapefruit seed extract (GES)**		**Goldenseal (D)**		**Goldenseal (Dl)**		**Noni juice (N)**		**Derum (Dr)**	
	GES 50%	GES+H	D50%	D+H	DL50%	DL+H	N50%	N+H	Dr50%	Dr+H
S. aureus	14.3±1.1	17.3±0.5	10.6±0.5	12.3±0.5	22.5±0.5	21.6±0.5	8±0	10±0	16.6±0.5	14±1
E.coli	8±0	8±0	8±0	NT	8±0	NT	8±0	8±0	11±0	12±1
MRSA	16±0	15.3±0.5	8±0	8±0	19.6±0.5	19.6±0.5	8±0	12.8±0.7	15.3±0.2	14±1
Candida inconspicua	8±0	NT	8±0	NT	14.3±0.5	14.3±1.1	8±0	NT	10±0	8±0
Candida rugosa	8±0	NT	8±0	NT	15.3±2	12.3±0.5	8±0	8±0	8±0	8±0

NT: not tested

H: Manuka honey 24+

2.3.6 The antibacterial activity of herbal extractions after being autoclaved

There was no significant difference between autoclaved and non-autoclaved activities for herbal extracts as shown in (Figs.2.13, 2.14 and 2.15) with exception of Derum, which showed an obvious difference between autoclaved and non-autoclaved activity against MRSA, *S. aureus* and *E.coli* (Figs.2.13, 2.14 and 2.15), as well as the effect of Goldenseal (D) which was removed after autoclaving against the Gram-positive bacteria MRSA and *S. aureus*.

Generally, autoclaving reduced the antibacterial activities of herbal extracts against all tested bacteria (Figs.2.13, 2.14 and 2.15). However, autoclaved and non-autoclaved activities for Goldenseal (DL) had the same effect against Gram-positive bacteria (Figs.2.13, 2.14), as did Grapefruit seed extract against all tested bacteria (Figs.2.13- 2.15).

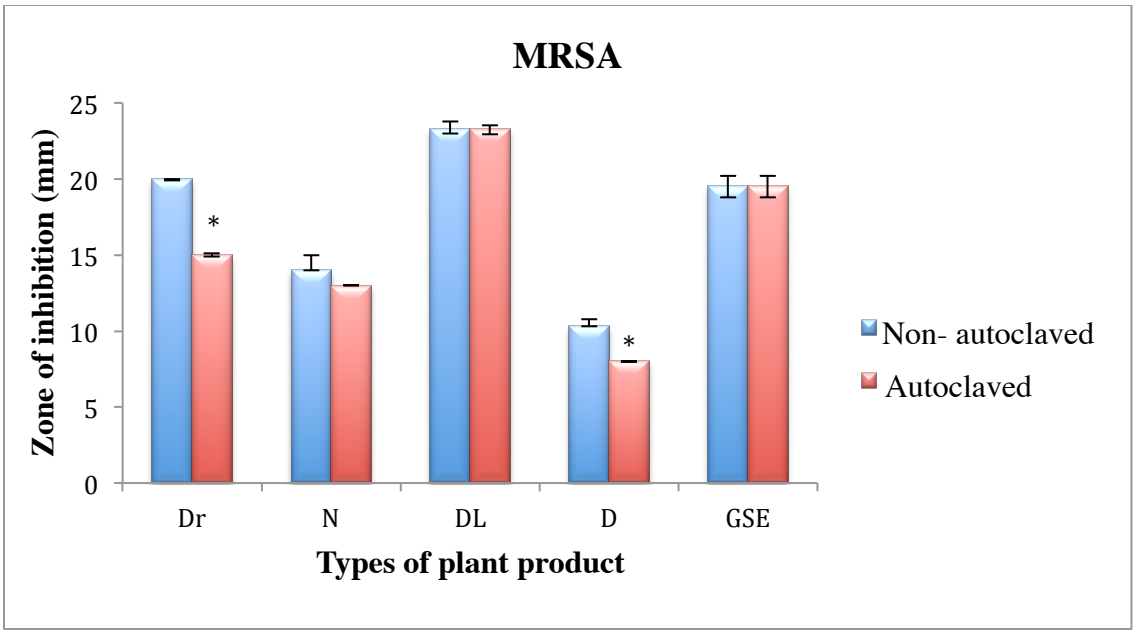

Figure 2.13 The antibacterial activity of autoclaved herbal extracts and non-autoclaved herbal extracts against MRSA determined by the agar diffusion method. The values are means of triplicates ± Standard Deviation (including the well (8.0mm)).

* Statistically significant differences at $P \leq 0.05$

Grapefruit seed extract= (GSE)

Goldenseal extract= (DL)

Noni juice= (N)

Goldenseal extract= (D)

Derum= (Dr)

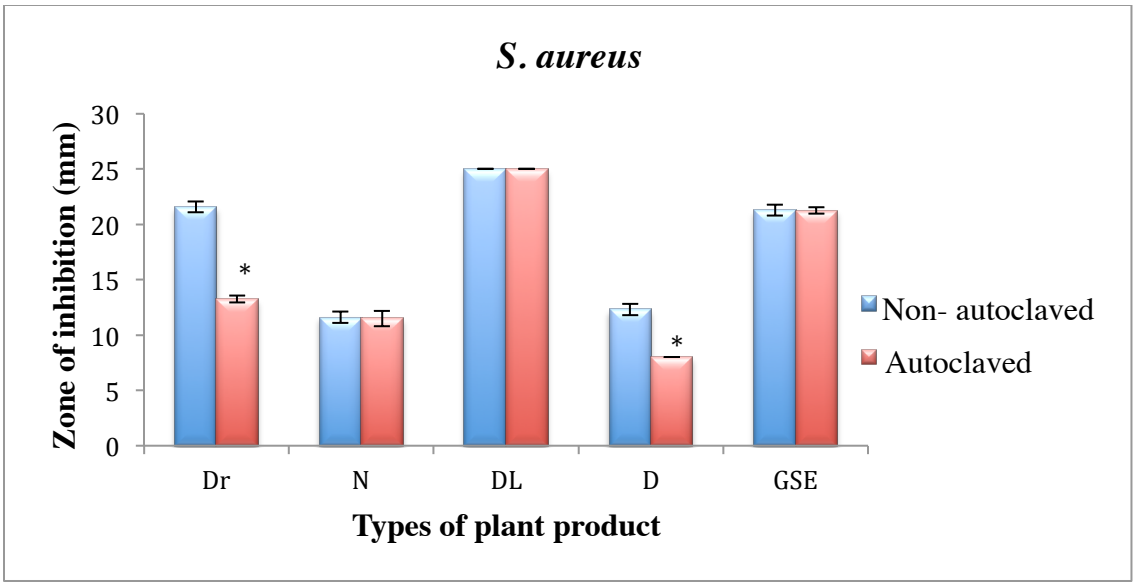

Figure 2.14 The antibacterial activity of autoclaved herbal extracts and non-autoclaved herbal extracts against *S. aureus* determined by the agar diffusion method. The values are means of triplicates ± Standard Deviation (including the well (8.0mm)).

* Statistically significant differences at $P \leq 0.05$

Grapefruit seed extract= (GSE)

Goldenseal extract= (DL)

Noni juice= (N)

Goldenseal extract= (D)

Derum= (Dr)

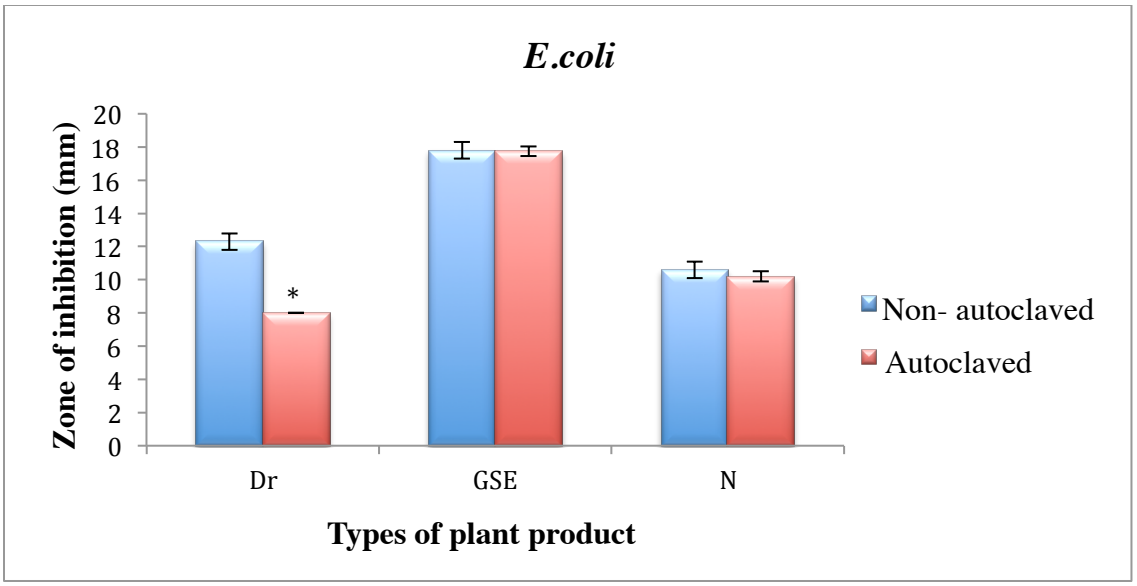

Figure 2.15 The antibacterial activity of autoclaved herbal extracts and non-autoclaved herbal extracts against *E.coli* determined by the agar diffusion method. The values are means of triplicates ± Standard Deviation (including the well (8.0mm)).

* Statistically significant differences at $P \leq 0.05$

Grapefruit seed extract= (GSE)

Noni juice= (N)

Derum= (Dr)

2.4 Discussion

Among the nine plant products, five were particularly effective against the bacteria and yeasts tested. The diversity of the antibacterial and antifungal effects of the plant products is attributed to a variety of factors, which act differently against the target organism, the age of the plant, part of the plant and seasonal variations may also be relevant. A study by Sibanda and Okoh (2007) showed that plant products often exhibit higher activity against Gram-positive bacteria than they do against Gram-negative species. An increase in the concentration of the plant products gave greater activity in all samples generally as was demonstrated by Choudhary *et al.* (2009) and both of the results presented here agree with these results. The minimum bactericidal or fungicidal concentration values for all of the plant products were higher than their minimum inhibitory concentration values, which suggest that plant products inhibited the growth of the study microorganisms but killed them at higher concentrations. Noni juice showed a paradoxical effect in that it produced a inhibition zone against *Candida rugosa,* but no MIC or MFC was detectable, even in high concentrations (100%). The reason for this is not immediately apparent, but may be due to differences relating to the use of agar as opposed to liquid medium. Moreover, there was no a statistically significant difference

between autoclaved and non-autoclaved activities for almost all herbal extracts in general; however, autoclaving reduced the antibacterial activities of Derum against all tested bacteria, as well as the effect of Goldenseal (D) against the Gram-positive bacteria MRSA and *S. aureus*, which agrees with the study by Hashemi *et al.* (2008) who recorded that autoclaving had a negative effect on antimicrobial activities of herbal extracts through decreasing these activities. However, the decline in the activities of herbal extracts by autoclaving was less than by acrodisc syringe filter; both methods having been used for sterilization (Hashemi *et al.,* 2008). Witkowska *et al.* (2013) noted that autoclaving reduced the antimicrobial properties of herbal extracts by inhibiting certain components as the result of their complete destruction, for example, when garlic and the spice plants in general are autoclaved at 100°C for 20 minutes (Azu *et al.,* 2006).

In conclusion, the results presented here show the antimicrobial activity of some herbal extracts against the bacteria and yeasts tested. Of these, Goldenseal and Grapefruit seed extract exhibited a wide spectrum of antibacterial effectiveness, a fact which demonstrates the importance of conducting a wide preliminary screening programme for antibacterial plant extracts.

Chapter Three: Antimicrobial Activity of Essential Oils

3.1 Introduction

3.1.1 Essential oil as alternative to antibiotics

The following essential oils have been shown to have antibiotic properties:

3.1.1.1 Kanuka oil

The scientific name of the Kanuka tree is *Kunzea ericoides* and its natural habitat is New Zealand. It is a member of the Myrtaceae family (Porter and Wilkins, 1998). Kanuka oil is known as Tea Tree oil due to early New Zealand settlers making tea from the leaves of this plant (Chen *et al*., 2016). Trees of the Kanuka usually reach more than 12 meters in height and their leaves are narrow, thick and less than 1.2 centimeters long with rounded tips (Van Vuuren *et al*., 2014). The essential oil is removed from the leaves and stems by steam distillation. For many decades, the local Maori and early European immigrants used this oil (Maddocks-Jennings *et al*., 2009) for a broad range of ailments including cold, coughs, skin conditions and poultices for back pain, burns and scalds, as a mouth wash for gum diseases and a tincture to promote sleep and reduce stress (Chen *et al*., 2016; Maddocks-Jennings *et al*., 2005; Wyatt *et al*., 2005). Clinically, Kanuka oil has antibacterial activity (Wyatt *et al*., 2005), analgesic, anti-inflammatory properties (Maddocks-Jennings *et al*., 2009). Van Vuuren *et al* (2014) noted

that while the antimicrobial activity of *K. ericoides* has been examined by disc diffusion, less interest has been shown in quantitative antimicrobial valuation. Chen *et al.* (2016) reported that Kanuka oil had fungicidal and bactericidal qualities against eight microbes, namely *Candida albicans, Malassezia furfur, Trichosporon mucoides, Candida tropica, Streptococcus mutans, Escherichia coli, Streptococcus sobrinus* and *Staphylococcus aureus*. The same authours found that due to its antimicrobial and anti-inflammatory properties, Kanuka oil should be considered for use as a therapeutic antibiotic, and food supplement. Maddocks-Jennings *et al.* (2005) found that a gargle or mouthwash including Kanuka oil could postpone the evolution of mucositis and decrease related health problems. Wyatt *et al.* (2005) reported that twig-derived extracts are less bioactive than the leaf extracts. In recent years, the antimicrobial action of Kanuka oil has been recognised and the Australian Standard (AS 2782-1985) shows that its two main components are (Figs.3.1 and 3.2):

1- 1,8-Cineole-which occurs in concentrations of less than 15% (Carson *et al*., 1995; Maddocks-Jennings *et al*., 2005; Lee *et al.,* 2013).
2- Terpinen-4-ol, the germicidal component, present at more than 30% (Carson *et al*., 1995; Maddocks-Jennings *et al*., 2005; Lee *et al*.,

2013).

Maddocks-Jennings *et al.* (2005) noted that there are differences in the constitution of plants depending on the geographical location or the age of the plant or season of harvesting. For instance, trees that are three years old have between 17% and 34% more monoterpenes (b-pinene, myrcene and a-pinene) than young trees which have less than 1%; as a result, such factors need to be taken into account for commercial production.

3.1.1.2 Peppermint oil

Peppermint oil from the plant *Mentha piperita* is being investigated as an antimicrobial agent; it is colorless and is extracted by steam distillation from the leaves of this perennial herb (Alankar, 2000). It belongs to the Labiatae family (Alankar, 2000). Peppermint and its oil are widely used in food, cosmetics, the flavoring industry and pharmaceutical applications (Alankar, 2000; Iscan *et al*., 2002; Singh *et al*., 2015). The oil is known for its painkilling, antispasmodic, antioxidant, decongestant, and anti-inflammatory effects (Meamarbashi and Rajabi, 2013); and also for the treatment of stomach ailments such as indigestion and gas problems (Alankar, 2000); colds, toothache, nausea, cramps, sore throats and cancers

(Singh *et al*., 2015). While India is considered the largest producer of Peppermint oil (Alankar, 2000), it is widely consumed throughout the world-more than any other essential oil (Alankar, 2000; Iscan *et al*., 2002; Tyagi and Malik, 2011). The main constituents of the oil include menthol, menthyl acetate, menthofuran and menthone (Grigoleit and Grigoleit, 2005; Singh *et al*., 2015); its activity being based on the presence of a high concentration of menthol (Singh *et al*., 2015; Bassole *et al.,* 2010). Tyagi and Malik (2011) reported that Peppermint oil *in vivo* and *in vitro* exhibits a variety of antimicrobial properties, notably biofilm-formation prevention against *Streptococcus pyogenes* and *Streptococcus mutans*. Singh *et al*. (2015) also showed that Peppermint oil exhibits antibacterial activities against Gram-positive and Gram-negative bacteria, as well as possessing antiviral and fungicidal properties.

3.1.1.3 Tea Tree oil (TTO)

Australian Tea Tree oil is extracted from the leaves of *Melaleuca alternifolia*, by steam distillation. It is also named as *Melaleuca* oil (Carson *et al*., 2002). Tea Tree oil contains some 100 volatile constituents (Budhiraja *et al*., 1999); terpinen-4-ol (fig 3.1) is considered to be the essential antimicrobial component and throughout history, TTO has been used for therapeutic purposes and as an additive to cosmetics (Carson *et al*.,

2006; Lee *et al*., 2013). It is also used to treat acne (Carson *et al*., 2002), oral candidiasis, tinea, cold sores (Hammer *et al*., 2012) and in the decrease of MRSA colonization in patients (Carson *et al*., 2002; Hammer *et al*., 2012; Loughlin *et al*., 2008). Dryden *et al.* (2004) showed that Tea Tree oil cures MRSA-related skin infections more effectively than does the standard medical treatment. Caelli *et al.* (2000) also showed that TTO was as effective as the standard MRSA medical treatment. The essential oil of *Melaleuca alternifolia* is anti-inflammatory, antifungal, antibacterial, and antiviral (Carson *et al*., 2002; Loughlin *et al.,* 2008).

3.1.1.4 Peru balsam

This is a tropical tree from the Fabaceae family (Pfutzner *et al*., 2003). The oil is derived from the bark of *Myroxolon balsamum* (Skypala *et al*., 2011). Peru balsam is an antiseptic (Pfutzner *et al*., 2003), which is used in foods, drinks, cosmetics and pharmaceutical products (Skypala *et al*., 2011). The oil smells like vanilla (Pfutzner *et al*., 2003) and its main components are vanilla, cinnamic acid, cinnamyl cinnamate, benzoic acid and benzyl benzoate (Skypala *et al*., 2011). Jasper *et al.* (1956) reported that Peru balsam oil showed considerable antibacterial activity against various pathogenic and non-pathogenic Gram-positive and Gram-negative bacteria. Also, Kavanaugh and Ribbeck (2012) found that oils of Peru balsam, cassia

and red thyme are more effective in destroying *S. aureus* and *Pseudomonas* biofilms than widely used antibiotics and could possibly replace the standard treatment approaches for biofilms.

3.1.1.5 Eucalyptus oil

This oil comes from the Myrtaceae family and is native to Tasmania (Kumar and Laxmidhar, 2011) and Southeast Australia (Abdossi *et al*., 2015). The Australian eucalyptus tree, also named as blue gum or Tasmanian blue gum, is one of the tallest trees in the world, reaching heights of 100 m (Boukhatem *et al*., 2014). The oil is extracted by the steam distillation method from dried leaves of eucalyptus (Nadjib *et al*., 2014). Essential oils from the Eucalyptus tree (*Eucalyptus globulus*) have been widely used in traditional medicines as anti-inflammatory, analgesic and antipyretic agent for the treatment of respiratory, colds and flu (Boukhatem *et al*., 2014) as well as in the treatment of skin rashes, influenza and chest pains, while the vapour can be used to combat inflammation (Damjanovic-vratnica *et al.*, 2011). It is also used in industries, pharmaceutical, cosmetics products and food, and for therapeutic purposes (Abdossi *et al.,* 2015; Boukhatem *et al.,* 2014). The main chemical components are α-pinene, β-myrcene and 1.8 cineole (Fig 3.2); and the other compounds are linalool, β-pinene, limonene, α-phellandrene, pinocarveol, α-terpineol, terpinen-4-ol,

and γ-terpinene (Boukhatem *et al.,* 2014; Damjanovic-vratnica *et al.,* 2011; Nadjib *et al.,* 2014). Clinically, the *Eucalyptus globulus* oil possesses antimicrobial activity, which is active against many common human pathogens such as *Escherchia coli, Staphylococcus aureus*, and *Streptococcus pyogenes*, spoilage bacteria and fungi (Damjanovic-vratnica *et al.,* 2011). As a result, it can be considered a beneficial alternative antimicrobial agent in the natural treatment of several infectious diseases (Damjanovic-Vratnica *et al.,* 2011).

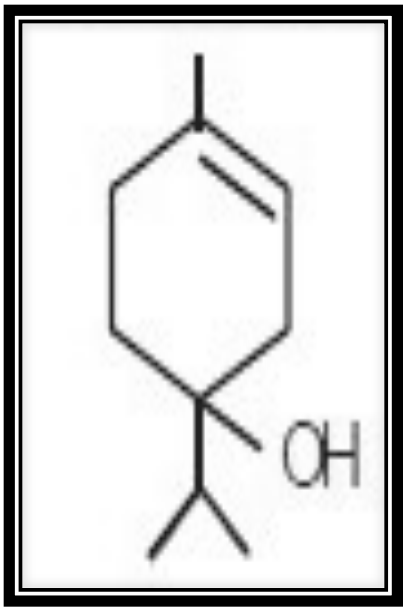

Figure 3.1 The chemical structure of terpinene-4-ol (Porel *et al.,* 2014).

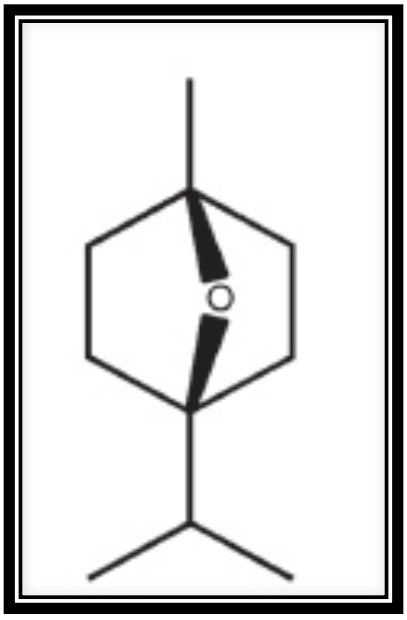

Figure 3.2 The chemical structure of 1,8 cineole (Neves and Camara, 2016).

The aim of the experimental work described in this chapter was to dedicate to a broad screening programme aimed at finding the most effective antimicrobial essential oils.

3.2 Materials and methods

3.2.1 Essential oil samples

Ten essential oils, listed in Table 3.1, were obtained from commercial suppliers; all samples were stored at room temperature until used.

Essential oil	
Scientific name	Common name
Prunus dulcis	Sweet Almond oil
Cucurbita pepo	Pumpkin seed oil
Origanum vulgare	Wild Oregano oil
Paullinia cupana	Guarana Seed oil
Helianthus annuus	Sunflower oil
Mentha piperita	Peppermint oil (T)
Eucalyptus globulus	Eucalyptus oil (E)
Kunzea ericoides	Kanuka oil (K)
Melaleuca alternifolia	Tea Tree oil (TT)
Myroxolon balsamum	Peru balsam oil (Pe)

Table 3.1 The ten essential oils (EOs) obtained from commercial products.

3.2.2 Essential oil sample preparation

Essential oil samples were generally used directly as purchased, although various sample concentrations from 5% to 100%v/v emulsion were also prepared. Tween 80 was added when necessary.

3.2.3 Test organisms

The following test microorganisms were used: *Staphylococcus aureus*, Methicillin-Resistant *S. aureus* MRSA, *Escherichia coli*, *Candida rugosa* and *Candida inconspicua*.

3.2.4 Inoculum preparation and turbidity standard

The inocula of susceptibility tests were adjusted to $1.5x10^8$ CFU/ml which reference to the (0.5 McFarland standards). Two to five isolated colonies from pure microbial culture were taken with a sterile loop and inoculated into a tube containing distilled, sterile water and mixed by using vortex until the mixture became homogeneous. The turbidity was measured by a spectrophotometer at 600 nm for bacteria, and at 580 nm for yeast. These suspensions were used within 30 minutes of preparation (Andrews, 2001).

3.2.5 Determination of microbial contamination of essential oils

In order to identify and detect any bacteria or fungi contaminating the essential oils chosen in this study, isolation of microorganisms from

samples was carried out using Nutrient agar and Saboraud Dextrose agar (incubated at 37°C under aerobic conditions for 24hours). Sabouraud Dextrose agar plates were incubated at 28°C under aerobic conditions for 48hours.

3.2.6 Agar diffusion assay

The plates were prepared using 20 ml of sterile media. A total of 0.1 ml of 0.5 McFarland standardised inoculum suspension of bacterial or yeast suspension was poured on each plate containing Muller-Hinton agar for bacteria or Saboraud Dextrose agar for yeasts. A sterile glass spreader was used to distribute the inoculum onto the surface. All plates were dried for 30 minutes and then wells, 8.0 mm in diameter, were cut from the culture media with a sterile metal cylinder, and then filled with 0.1 ml; the test samples had concentrations of 100%, 80%, 50%, 25% and 5%v/v. Sterile water served as a negative control. After a 30 min pre-diffusion time interval, the Petri dishes were subsequently incubated at 37°C for 24 hours for bacteria and at 28°C for 48hours for yeasts and the inhibition zone around each well was measured in mm, including the well (8.0mm). The result was then recorded. All experiments were carried out in triplicate. The effectiveness of antimicrobial essential oils was determined using the well diffusion assay.

3.2.7 Determination of the minimum inhibitory concentration (MIC)

The minimal inhibitory concentration (MIC) was determined by the macro broth dilution method. A series dilution of each essential oil was prepared in (0.05% Tween 80), to produce a concentration range from 0.4 % to 50% v/v. Each tube-contained 2ml Mueller-Hinton broth medium for bacteria or Saboraud Dextrose broth for yeasts with samples. Each tube received 0.02 ml of bacterial or yeasts suspension adjusted to 0.5 McFarland turbidity standards. Controls without essential oils, without bacterial or yeasts inoculum or with essential oils only were also included. The mixtures were homogenized using a Vortex mixer for 2 minutes, and the tubes were then incubated in 37°C with shaking at 250 rpm for 24 hours for bacteria and 48 hours in 28°C with shaking at 180 rpm for yeasts. The first tube in the above series with no visible growth was marked as the MIC.

3.2.8 Determination of the minimum bactericidal concentrations

Bacterial culture (0.01ml) was taken from the tubes with no growth and plated onto Mueller–Hinton agar then incubated overnight at 37°C to determine MBCs. The lowest concentration, which showed no growth on the agar, was defined as the MBC.

3.2.9 Determination of the minimum fungicidal concentrations

After determining the MICs, 0.01ml was taken from the tubes with no growth and plated onto Saboraud Dextrose agar and then incubated at 28°C; MFCs were recorded after 48 hours. The MFC was defined as the lowest concentration that resulted in no growth in these plates.

3.2.10 Determination of the effect of essential oils after mixing with honey

The antimicrobial effect of essential oils, after mixing with honey, was evaluated using the agar diffusion method; the plates were prepared using 20 ml of sterile media. A total of 0.1 ml of bacterial or yeast suspension was poured on each plate containing Muller-Hinton agar for bacteria and Sabouraud Dextrose agar for yeasts. A sterile glass spreader was used to distribute the inoculum on the surface of agar. All plates were allowed to dry for 30 minutes and wells (8.0 mm in diameter) were cut from the culture media using a sterile metal cylinder, and then filled with 0.05 ml 24+ Manuka honey and 0.05 ml of the test samples. Each well received 0.1ml of mixture. After a 30 min pre-diffusion time interval, the Petri dishes were subsequently incubated at 37°C for 24 hours for bacteria and at 28°C for 48 hours for yeasts, and the inhibition zone around each well was measured in mm, including the well (8.0mm). The result was then recorded.

Essential oils alone (0.1ml of 50% v/v emulsion) were used as the control.

3.2.11 The antibacterial activity of essential oils after being autoclaved

Each essential oil was autoclaved at 120°C for 15 minutes, and then its antibacterial activity was determined against all the tested bacteria by use of well diffusion assay. Non- autoclaved samples of essential oils were used as the control.

3.2.12 Statistical Analysis

All observations were presented as Mean ± SD (Standard Deviation). The data were analysed by IBM Corp© 24.0. One way ANOVA was performed to compare if there was a significance difference of the inhibition zone values measured between the different essential oils against the test organisms. $P \leq 0.05$ was considered as statistically significant. Tukey-Post-Hoc test confirmed the pairwise comparisons.

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3.3 Results

3.3.1 Determination of the microbial contamination of essential oils

No microbial contamination was found in any of the samples of essential oils.

3.3.2 Agar diffusion assay

Ten essential oils were evaluated for antimicrobial activity using the agar diffusion assay against Gram-negative bacteria and Gram-positive bacteria including methicillin-resistant *S. aureus* (MRSA) as well as the yeast species. Half of these essential oils showed measurable inhibitory activity against at least four of the types of microorganisms tested, while the other half exhibited no detectable inhibitory activity. The antimicrobial activities of the essential oils are presented in Tables 3.2, 3.3, 3.4, 3.5 and 3.6.

Generally, Kanuka oil showed the most potent activity against *Staphylococcus aureus*, and there is a statistically significant difference between Kanuka oil and other essential oils ($P \leq 0.006$) (Table 3.2) followed by Eucalyptus oil ($P \leq 0.006$) (Fig.3.6). Also, an intermediate inhibition zone was detected for Tea Tree oil ($P \leq 0.02$), and Peppermint oil ($P \leq 0.02$), against *Staphylococcus aureus* (Table 3.2), while Peru balsam oil exhibited the least inhibitory activity against *Staphylococcus aureus* and there was a

statistically significant difference between Peru balsam oil and other essential oils ($P \leq 0.001$) (Table 3.2).

In the case of *E.coli*, Tea Tree oil showed the most marked antibacterial activity, and there was a statistically significant difference between Tea Tree oil and other essential oils ($P \leq 0.03$) and Kanuka oil was the next most inhibitory ($P \leq 0.03$) (Table 3.3). Moreover, Eucalyptus oil showed a moderate inhibition zone ($P \leq 0.003$) (Table 3.3) (Fig.3.7). However, Peppermint oil and Peru balsam oil (Table 3.3) did not exhibit any antimicrobial activity, even at the highest concentration (100%).

Kanuka oil (Fig.3.4) generally showed a higher antibacterial effect than other oils against MRSA, and there was a statistically significant difference between Kanuka oil and other essential oils ($P \leq 0.05$) (Table 3.4); Eucalyptus oil showed the next highest activity ($P \leq 0.05$) (Table 3.4) (Fig.3.5), followed by Tea Tree oil ($P \leq 0.005$) (Table 3.4), whereas Peppermint oil and Peru balsam oil showed a moderate inhibitory activity against MRSA (Table 3.4). Tea Tree oil was seen to be the most effective oil with the biggest zone (43±1) against *Candida inconspicua*, and there was a statistically significant difference between Tea Tree oil and other essential oils ($P = 0.001$) (Table 3.5). However, significant zones of

inhibition and strong antifungal activity were detected for Peppermint oil and Tea Tree oil against *Candida rugosa*, and there was a statistically significant difference between the antimicrobial activity of Tea Tree oil and Peppermint oil (*P=0.05*) (Table 3.6).

An increase in the concentration of the essential oils generally produced greater inhibition as shown by the diameter of zone of inhibition. In the case of Tea Tree oil (Fig. 3.3), the 80% concentration gave larger inhibition zones against *S. aureus* than the 100% (Table 3.2) (i.e. a paradoxical effect), as did Eucalyptus oil against *E.coli* (Table 3.3) and Peppermint oil and Tea Tree oil against *Candida rugosa* (Table 3.6).

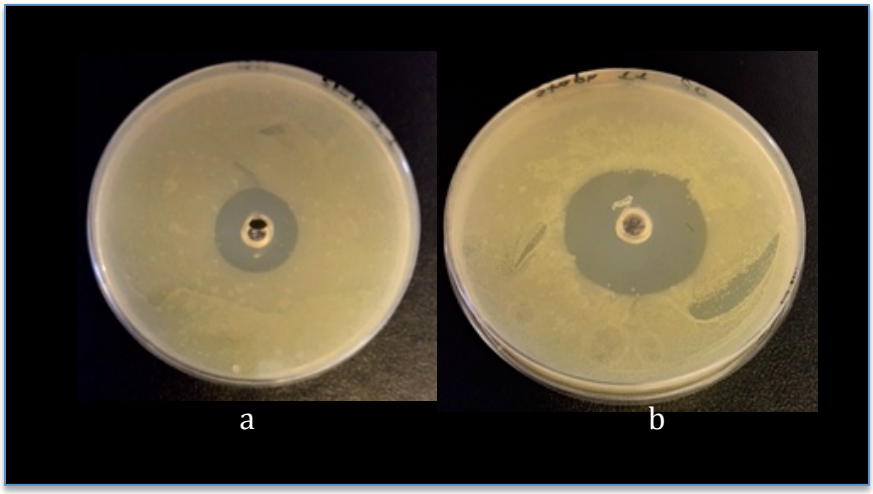

Figure 3.3 Agar diffusion assay for Tea Tree oil at various concentrations: 100% and 80%. Plates seeded with *S. aureus:* (a) zone of inhibition caused by 100 % and (b) zone of inhibition caused by 80%.

Figure 3.4 Agar diffusion assay for Kanuka oil against MRSA caused by 100%.

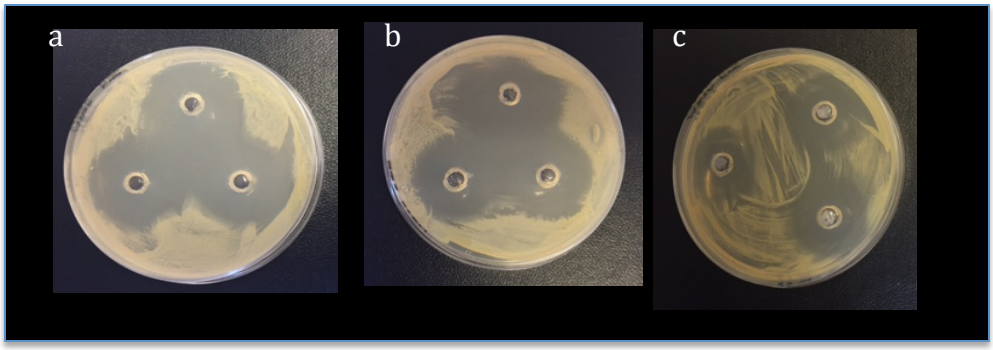

Figure 3.5 Agar diffusion assay for Eucalyptus oil at various concentrations: 100%, 80% and 50%. Plates seeded with MRSA*:* (a) zone of inhibition caused by 100 %, (b) zone of inhibition caused by 80% and (c) zone of inhibition caused by 50%.

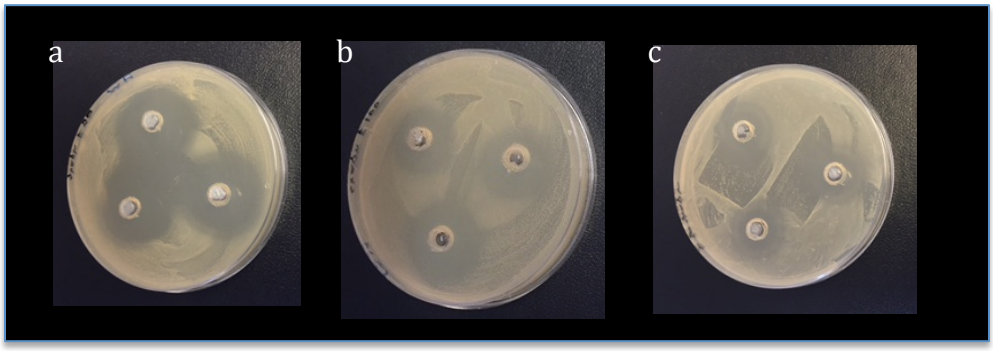

Figure 3.6 Agar diffusion assay for Eucalyptus oil at various concentrations: 100%, 80% and 50%. Plates seeded with *S. aureus:* (a) zone of inhibition caused by 100 %, (b) zone of inhibition caused by 80% and (c) zone of inhibition caused by 50%.

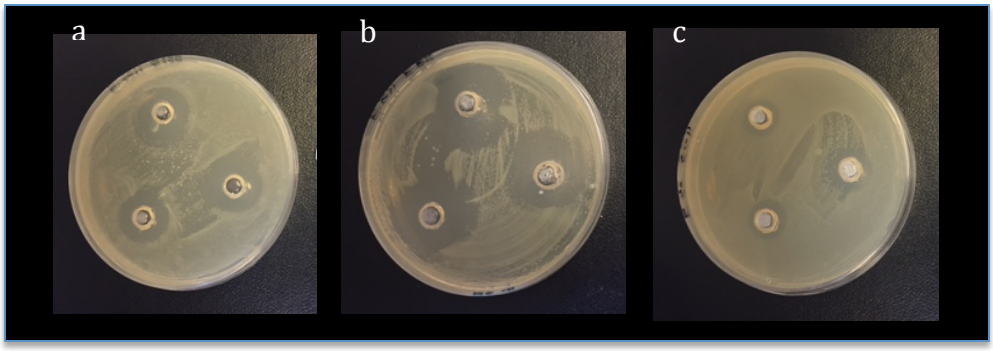

Figure 3.7 Agar diffusion assay for Eucalyptus oil at various concentrations: 100%, 80% and 50%. Plates seeded with *E. coli:* (a) zone of inhibition caused by 100 %, (b) zone of inhibition caused by 80% and (c) zone of inhibition caused by 50%.

Table 3.2 Effect of different concentrations of various essential oils against ***S. aureus*** determined by use of the agar diffusion assay. Means of triplicates (diameter mm including well (8.0 mm)) ± Standard Deviation.

	Essential Oil concentrations				
Type of Essential Oils	**100%**	**80%**	**50%**	**25%**	**5%**
Kanuka oil*	28.5±0.7	23.3±1.5	20.5±0.5	17.3±0.5	8±0
Peru balsam oil*	17±0	15.6±1.1	14±1	8±0	8±0
Eucalyptus oil*	26±1	21±1.7	15.9±0.8	8±0	8±0
Tea Tree oil*	22.3±0.5	34.6±1.5	23.6±0.5	20±1	8±0
Peppermint oil*	20.3±0.7	16±1	14±1	8±0	8±0
Sunflower oil	8±0	8±0	8±0	8±0	8±0
Sweet Almond oil	8±0	8±0	8±0	8±0	8±0
Pumpkin seed oil	8±0	8±0	8±0	8±0	8±0
Wild Oregano oil	8±0	8±0	8±0	8±0	8±0
Guarana Seed oil	8±0	8±0	8±0	8±0	8±0

(*Statistically significant differences at $P \leq 0.05$)

Table 3.3 Effect of different concentrations of various essential oils against ***E.coli*** determined by use of the agar diffusion assay. Means of triplicates (diameter mm including well (8.0 mm)) ± Standard Deviation.

	Essential Oil concentrations				
Type of Essential Oils	**100%**	**80%**	**50%**	**25%**	**5%**
Kanuka oil*	21.6±1.1	21.3±0.5	16.3±1.5	9±0	8±0
Peru balsam oil	8±0	8±0	8±0	8±0	8±0
Eucalyptus oil*	19.1±0.2	22.5±1.3	15.5±0.5	8±0	8±0
Tea Tree oil*	23.3±0.5	19.8±1.6	18±1	8±0	8±0
Peppermint oil	8±0	8±0	8±0	8±0	8±0
Sunflower oil	8±0	8±0	8±0	8±0	8±0
Sweet Almond oil	8±0	8±0	8±0	8±0	8±0
Pumpkin seed oil	8±0	8±0	8±0	8±0	8±0
Wild Oregano oil	8±0	8±0	8±0	8±0	8±0
Guarana Seed oil	8±0	8±0	8±0	8±0	8±0

(*Statistically significant differences at $P \leq 0.05$)

Table 3.4 Effect of different concentrations of various essential oils against **MRSA** determined by use of the agar diffusion assay. Means of triplicates (diameter mm including well (8.0 mm)) ± Standard Deviation.

	Essential Oil concentrations				
Type of Essential Oils	**100%**	**80%**	**50%**	**25%**	**5%**
Kanuka oil*	42±1	32.3±0.2	25.3±0.2	16.3±0.5	8±0
Peru balsam oil	16.1±0.2	13.6±1.1	12.6±1.1	10±1	8±0
Eucalyptus oil*	34.3±1.1	30.3±1.5	18.3±1.5	8±0	8±0
Tea Tree oil*	21.3±1.	16±1	13.8±0.7	8±0	8±0
Peppermint oil	17.3±1.2	13.6±0.2	15.3±0.5	13.5±1.5	8±0
Sunflower oil	8±0	8±0	8±0	8±0	8±0
Sweet Almond oil	8±0	8±0	8±0	8±0	8±0
Pumpkin seed oil	8±0	8±0	8±0	8±0	8±0
Wild Oregano oil	8±0	8±0	8±0	8±0	8±0
Guarana Seed oil	8±0	8±0	8±0	8±0	8±0

(*Statistically significant differences at $P \leq 0.05$)

Table 3.5 Effect of different concentrations of various essential oils against ***Candida inconspicua*** determined by use of the agar diffusion assay. Means of triplicates (diameter mm including well (8.0 mm)) ± Standard Deviation.

	Essential Oil concentrations				
Type of Essential Oils	**100%**	**80%**	**50%**	**25%**	**5%**
Kanuka oil	23.3±1.5	15±1	12.3±0.5	14.3±0.5	11.3±0.5
Peru balsam oil	20±1	20.1±1.8	17.6±1.5	16.1±0.2	8±0
Eucalyptus oil	25.5±0.7	23±1.4	15.3±0.5	8±0	8±0
Tea Tree oil*	43±1	38.6±1.1	24.6±0.5	22.6±1.1	8±0
Peppermint oil	25.1±0.7	19.3±1	16.6±1.1	12.6±1.1	8±0
Sunflower oil	8±0	8±0	8±0	8±0	8±0
Sweet Almond oil	8±0	8±0	8±0	8±0	8±0
Pumpkin seed oil	8±0	8±0	8±0	8±0	8±0
Wild Oregano oil	8±0	8±0	8±0	8±0	8±0
Guarana Seed oil	8±0	8±0	8±0	8±0	8±0

(*Statistically significant differences at $P \leq 0.05$)

Table 3.6 Effect of different concentrations of various essential oils against ***Candida rugosa*** determined by use of the agar diffusion assay. Means of triplicates (diameter mm including well (8.0 mm)) ± Standard Deviation.

	Essential Oil concentrations				
Type of Essential Oils	**100%**	**80%**	**50%**	**25%**	**5%**
Kanuka oil	23.6±1.5	15.3±0.5	13.3±1.1	11.6±1.5	8±0
Peru balsam oil	22±1.7	20.3±1.1	19.3±1.1	17.5±0.7	8±0
Eucalyptus oil	29.1±0.7	22±0.2	15±1	8±0	8±0
Tea Tree oil	30.5±1.4	33.5±0.7	27±1.4	24±1.4	10.6±1.1
Peppermint oil*	34±0.7	41.5±0.7	21.6±1.1	19.8±1.2	8±0
Sunflower oil	8±0	8±0	8±0	8±0	8±0
Sweet Almond oil	8±0	8±0	8±0	8±0	8±0
Pumpkin seed oil	8±0	8±0	8±0	8±0	8±0
Wild Oregano oil	8±0	8±0	8±0	8±0	8±0
Guarana Seed oil	8±0	8±0	8±0	8±0	8±0

(*Statistically significant differences at $P \leq 0.05$)

3.3.3 Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC (Fig. 3.8) and MBC values of five essential oils against *E.coli, S. aureus* and methicillin-resistant *S. aureus* (MRSA), are shown in Figures 3.9, 3.10 and 3.11. The MIC values for all essential oils were lower than their MBC values generally. It was noticeable that the Kanuka oil recorded the lowest MIC values (0.4%v/v) against all Gram-positive bacteria (Fig. 3.9 and 3.10), whereas Kanuka oil inhibited the growth of Gram-negative bacteria at highest MIC values (Figure 3.11). Additionally, Eucalyptus oil and Tea Tree oil recorded the strongest bacteriostatic effect against *E.coli* at MIC value (0.8%v/v) as shown in Figure 3.11. Generally, Tea Tree oil demonstrated the strongest bactericidal activity against MRSA (Fig.3.9). Kanuka oil displayed the strongest bactericidal activity against *S. aureus* (Fig.3.10), while Tea Tree oil recorded the strongest bactericidal activity against *E.coli* (1.65%v/v) (Fig.3.11). Among EOs, Peru balsam oil showed the weakest antibacterial activity and, not surprisingly, had the largest MICs and MBCs (Fig.3.12 and 3.13).

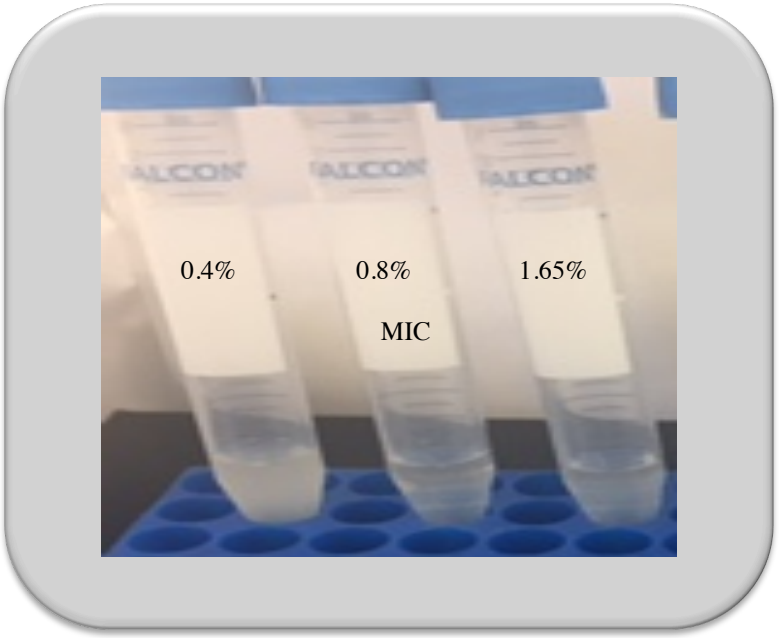

Figure3.8 A minimal inhibitory concentration (MIC)(%v/v) determined by macro dilution method for Eucalyptus oil against MRSA. The tube to the left indicates bacterial growth; the tubes to the right are clear, indicating no growth. The MIC was 0.8% v/v.

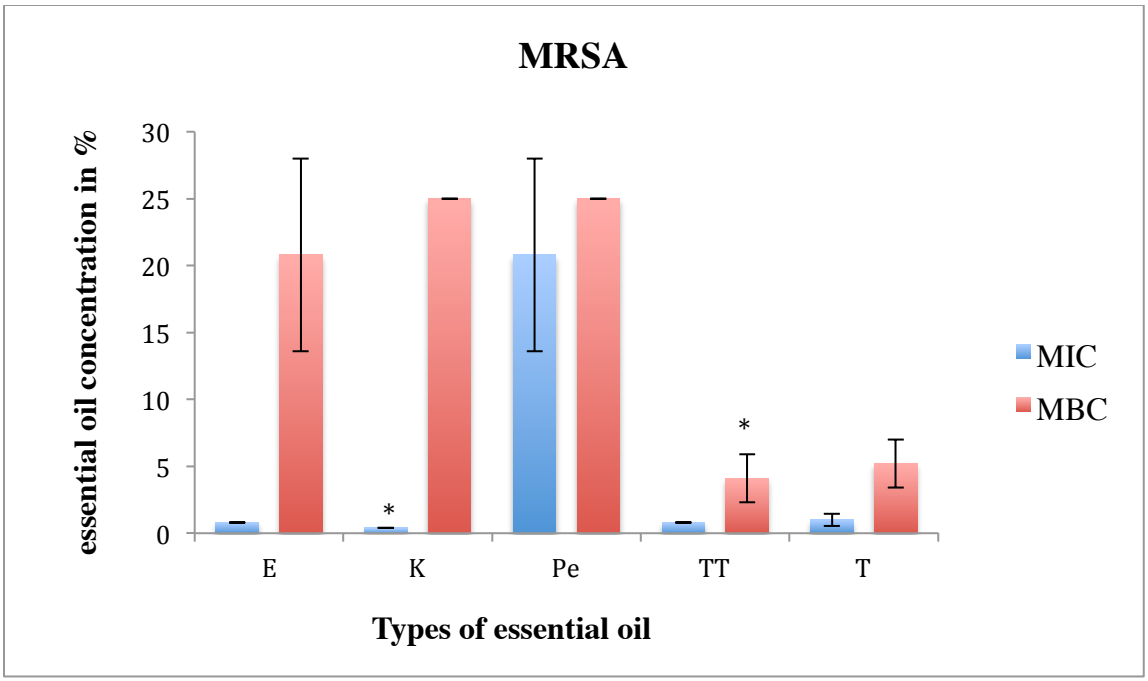

Figure 3.9 Minimum inhibitory concentrations (MICs) (%v/v), determined by the macro broth dilution assay, and minimum bactericidal concentrations (MBCs) of different essential oils against MRSA. The values are demonstrated as means of triplicates ± Standard Deviation.

* Statistically significant differences at $P \leq 0.05$.

There was not a statistically significant difference between K and E, TT and T in MIC group, as did TT and T in MBC group ($P \geq 0.05$).

Kanuka oil= (K)

Peru balsam oil= (Pe)

Eucalyptus oil= (E)

Tea Tree oil= (TT)

Peppermint oil= (T)

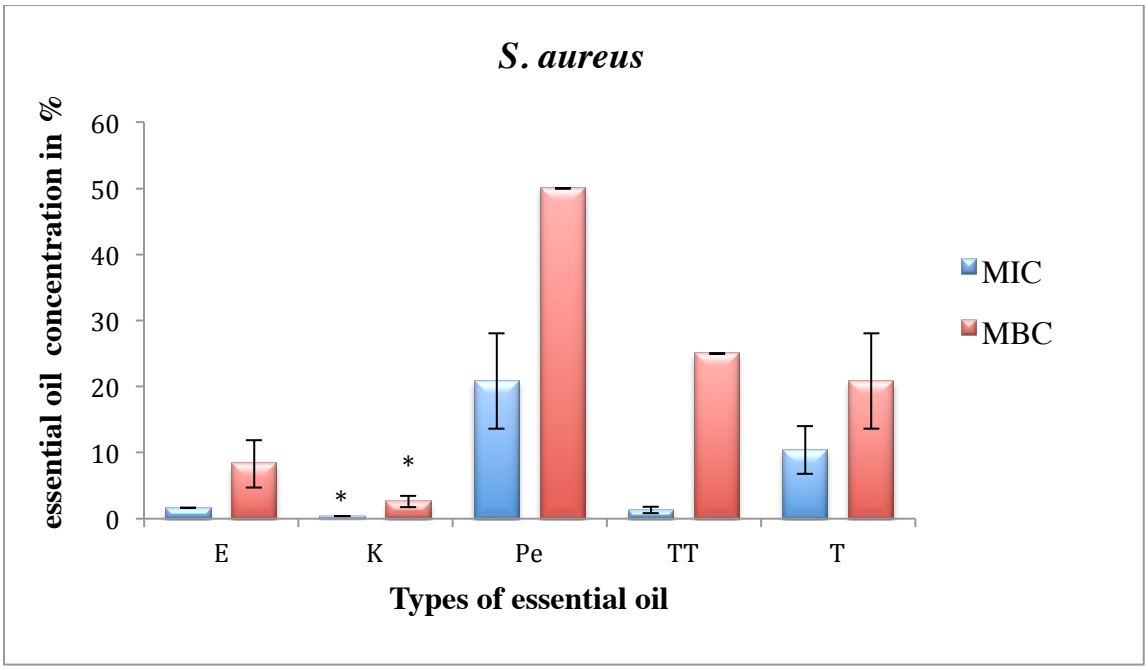

Figure 3.10 Minimum inhibitory concentrations (MICs) (%v/v), determined by the macro broth dilution assay, and minimum bactericidal concentrations (MBCs) of different essential oils against *S. aureus*. The values are demonstrated as means of triplicates ± Standard Deviation.

* Statistically significant differences at $P \leq 0.05$.

There was not a statistically significant difference between K and E, as well as K and TT in MIC group, and between K and E in MBC group ($P \geq 0.05$).

Kanuka oil= (K)

Peru balsam oil= (Pe)

Eucalyptus oil= (E)

Tea Tree oil= (TT)

Peppermint oil= (T)

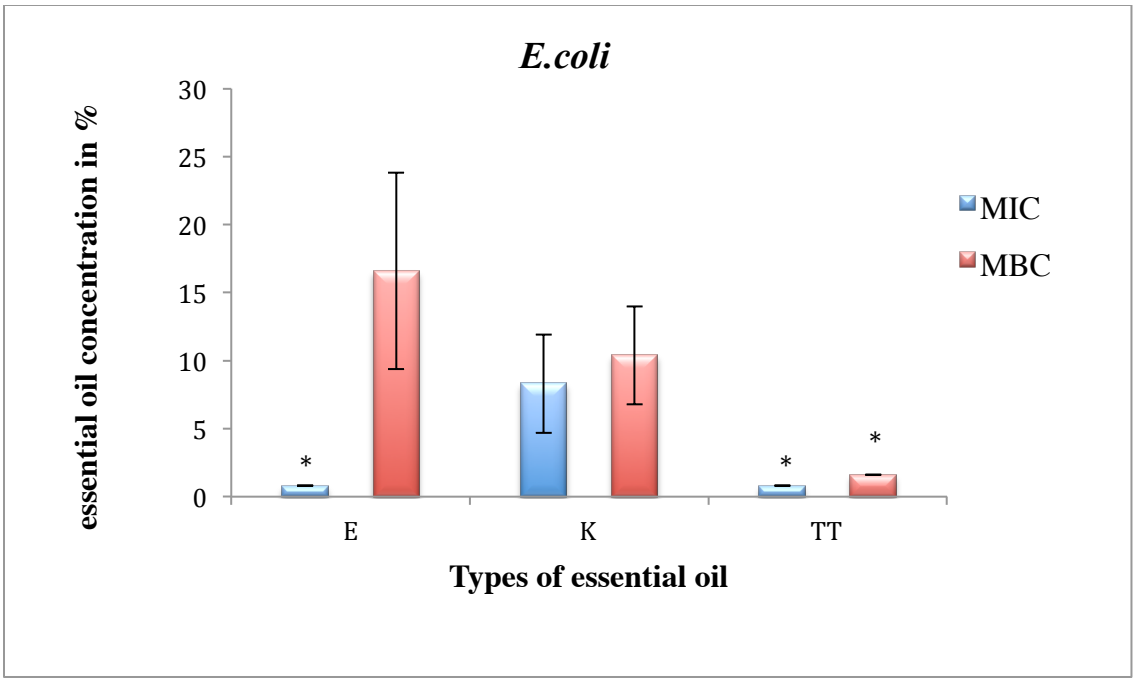

Figure 3.11 Minimum inhibitory concentrations (MICs) (%v/v), determined by the macro broth dilution assay, and minimum bactericidal concentrations (MBCs) of different essential oils against *E.coli.* The values are demonstrated as means of triplicates ± Standard Deviation.

* Statistically significant differences at $P \leq 0.05$

There was not a statistically significant difference between TT and E in MIC group ($P \geq 0.05$).

Kanuka oil= (K)

Eucalyptus oil= (E)

Tea Tree oil= (TT)

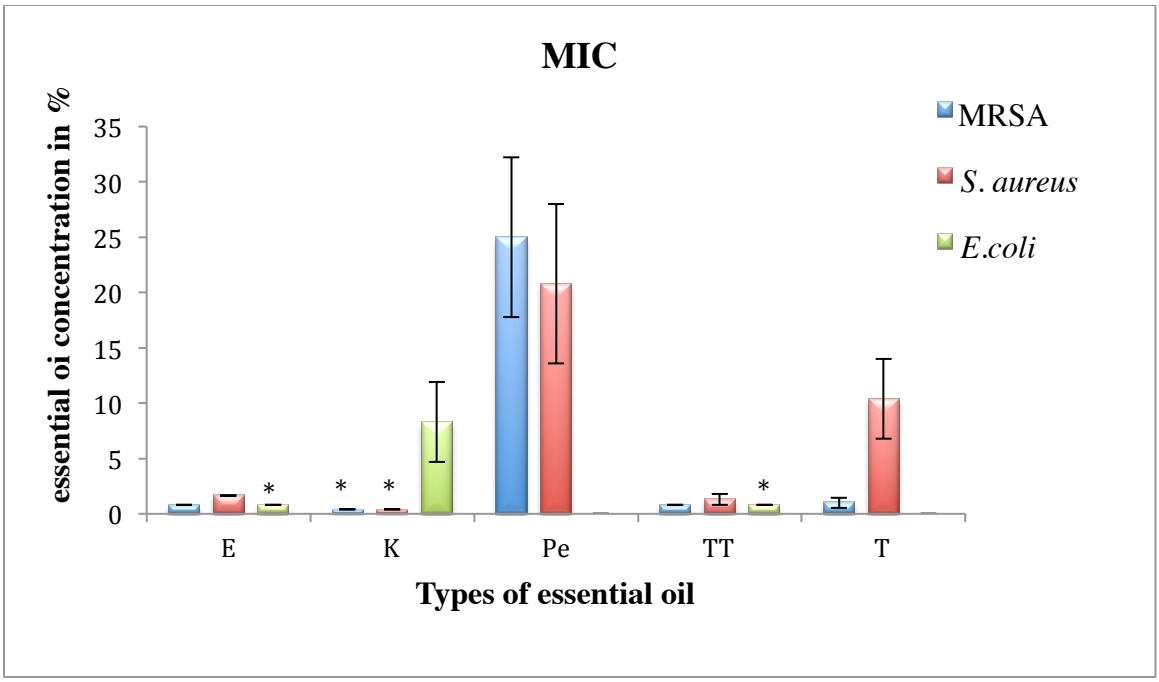

Figure 3.12 The minimum inhibitory concentrations (MICs) (%v/v) of essential oils against various pathogenic bacteria. Determined by the macro broth dilution assay. The values are demonstrated as means of triplicates ± Standard Deviation.

* Statistically significant differences at $P \leq 0.05$

There was not a statistically significant difference between K and E, TT and T in MRSA group, as well as K and E and K and TT in *S. aureus* group, also TT and E in *E. coli* group ($P \geq 0.05$).

Kanuka oil= (K)

Peru balsam oil= (Pe)

Eucalyptus oil= (E)

Tea Tree oil= (TT)

Peppermint oil= (T)

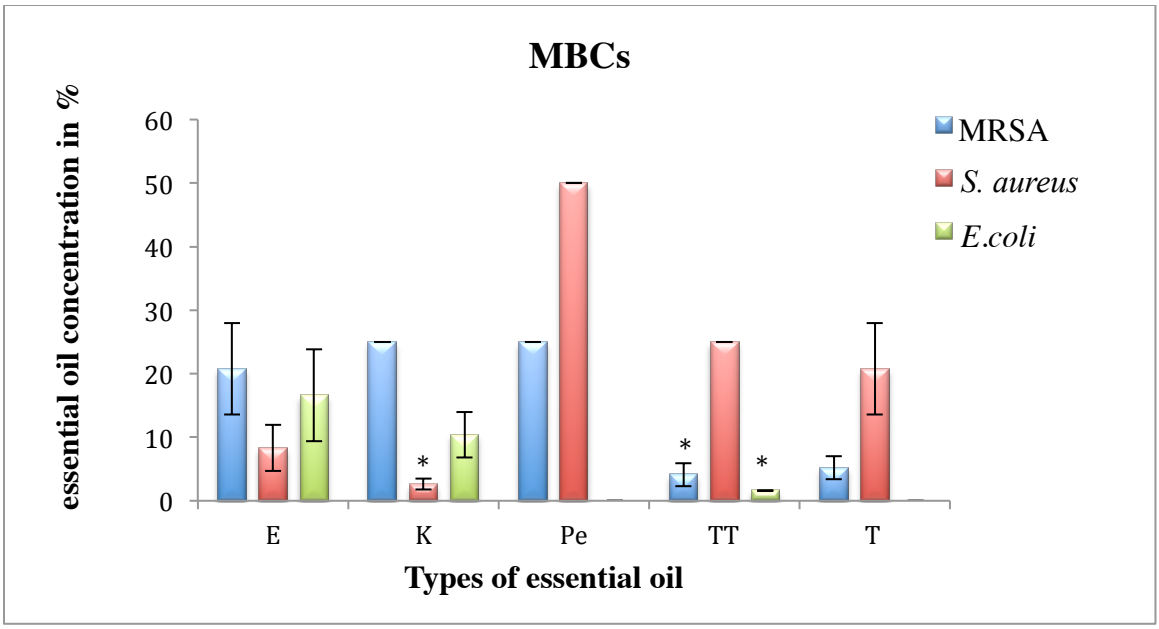

Figure 3.13 The minimum bactericidal concentrations (MBCs) of essential oils against various pathogenic bacteria. The values are demonstrated as means of triplicates ± Standard Deviation.

* Statistically significant differences at $P \leq 0.05$.

There was not a statistically significant difference between TT and T in MRSA group, as well as K and E in *S. aureus* group ($P \geq 0.05$).

Kanuka oil= (K)

Peru balsam oil= (Pe)

Eucalyptus oil= (E)

Tea Tree oil= (TT)

Peppermint oil= (T)

3.3.4 Determination of the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

The MIC and MFC values of five essential oils were determined against two types of yeasts (*Candida inconspicua, Candida rugosa*). These MIC values are shown in Figure 3.14. Generally, MFC values were higher than MIC values in almost all results. Tea Tree oil and Peppermint oil effectively inhibited the growth of both yeasts at the lowest MIC values (0.8%v/v)(Figure 3.14). Otherwise, Kanuka oil recorded the highest MIC values with both yeasts; as did Eucalyptus oil against *Candida rugosa* (Figure 3.14). Peppermint oil showed the most marked fungicidal effect against both yeasts tested at MFC value (0.8%v/v) as shown in Figure 3.15. However, Tea Tree oil showed the strongest fungicidal effect against *Candida inconspicua* only (Figure 3.15). In contrast, Kanuka oil had the highest MFC value with the weakest fungicidal effect against both yeasts (Figure 3.15).

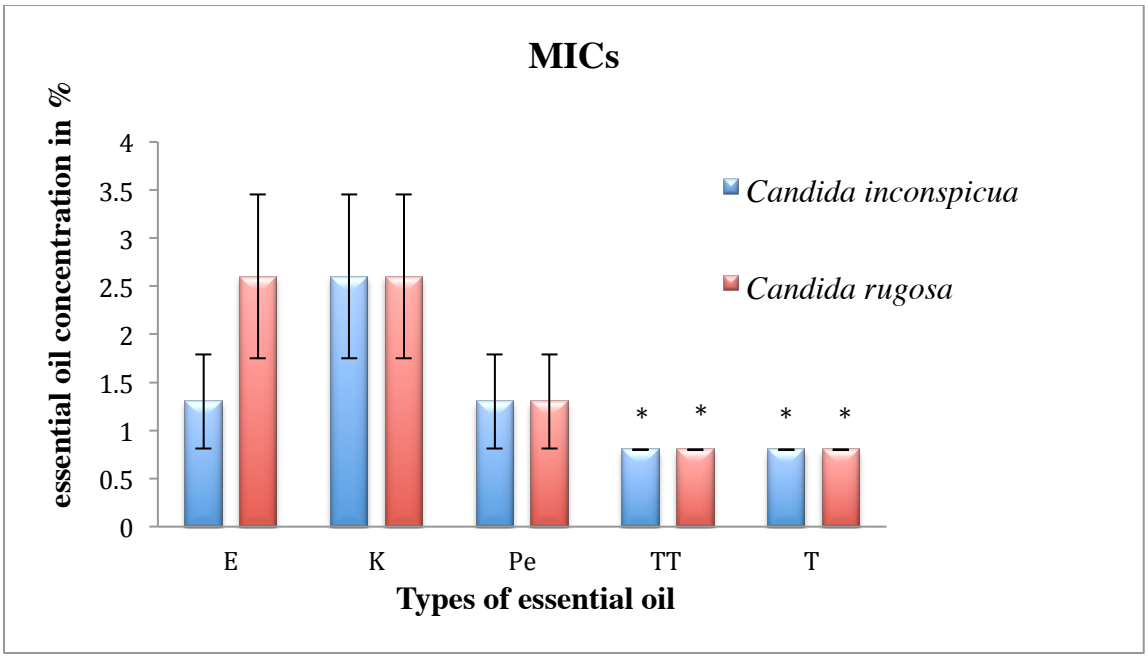

Figure 3.14 The minimum inhibitory concentrations (MICs) (%v/v) of essential oils against pathogenic yeasts. Determined by the macro broth dilution method. The values are demonstrated as means of triplicates ± Standard Deviation.

* Statistically significant differences at P≤0.05.

There was not a statistically significant difference between T and TT as well as T or TT and Pe in both groups, as well as T or TT and E in *Candida inconspicua* group (P≥0.05).

Kanuka oil= (K)

Peru balsam oil= (Pe)

Eucalyptus oil= (E)

Tea Tree oil= (TT)

Peppermint oil= (T)

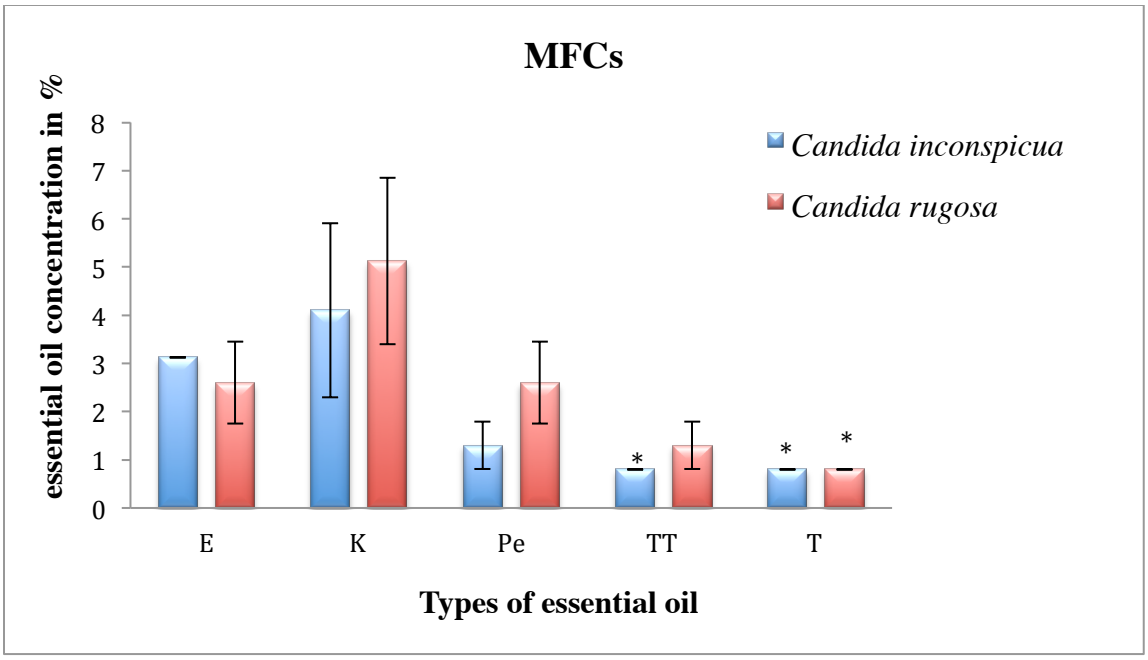

Figure 3.15 The minimum fungicidal concentrations (MFCs) of essential oils against pathogenic yeasts. The values are demonstrated as means of triplicates ± Standard Deviation.

* Statistically significant differences at P≤0.05.

There was not a statistically significant difference between T and TT, Pe and E in *Candida rugosa* group. Also, there was not a statistically significant difference between T and TT as well as T and Pe in *Candida inconspicua* group (P≥0.05).

Kanuka oil= (K)

Peru balsam oil= (Pe)

Eucalyptus oil= (E)

Tea Tree oil= (TT)

Peppermint oil= (T)

3.3.5 Determination of the effect of essential oils after mixing with honey

The antimicrobial activities of mixing 24+ Manuka honey and essential oils were evaluated by using the agar well diffusion assay. The results generally showed that the inhibitory effect of combinations was increased in comparison with each essential oil individually (Table 3.7). However, Peppermint oil showed a decrease in inhibition zone against *Candida rugosa* after being mixed with Manuka honey 24+, as did Peru balsam oil against *Candida inconspicua* (Table 3.7). Interestingly, Peru balsam oil presented the same effect after and before mixing with honey against *Candida rugosa* (Table 3.7). Furthermore, Tea Tree oil showed a decrease in diameter of inhibition zone after mixing with Manuka honey 24+ against *S. aureus* (Figure 3.16) and both yeasts (Table 3.7), as well as Eucalyptus oil against *E.coli* (Table 3.7).

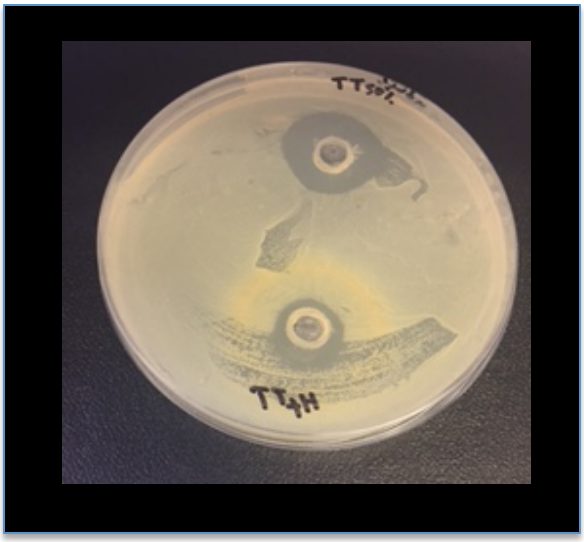

Figure 3.16: Comparison of inhibitions zone for Tea Tree oil before and after mixing with Manuka honey 24+; plate seeded with *S. aureus*.

Table 3.7 Susceptibility pattern of the combinations of Manuka honey and essential oils against different microorganisms determined by the well agar diffusion method. The values are means of triplicates (diameter mm including well (8.0 mm)) ± Standard Deviation.

Bacteria	**Kanuka oil**		**Peru balsam oil**		**Eucalyptus oil**		**Peppermint oil**		**Tea Tree oil**	
	K (50%)	K+H	Pe (50%)	Pe+H	E (50%)	E+H	T (50%)	T+H	TT (50%)	TT+H
S. aureus	20.5±0.5	26±2	14±1	15.1±0.2	15.9±0.8	21.6±0.5	14±1	17.8±0.2	23.6±0.5	17.3±0.5
E.coli	16.3±1.5	17.3±0.5	8±0	NT	15.5±0.5	14.3±0.5	8±0	NT	18±1	18.3±0.2
MRAS	25.3±0.2	28.3±0.5	12.6±1.1	17.6±0.5	18.3±1.5	22.5±0.5	15.3±0.5	28.3±0.5	13.8±0.7	20.1±0.2
Candida inconspicua	12.3±0.5	15.8±0.2	17.6±1.5	16.3±0.5	15.3±0.5	17±1	16.6±1.1	21±1	24.6±0.5	18.3±0.5
Candida rugosa	13.3±1.1	20.3±0.5	19.3±1.1	19.3±0.5	15±1	15.6±0.5	21.6±1.1	18±1	27±1.4	15.3±0.5

K: Kanuka oil PE: Peru balsam oil

E: Eucalyptus oil TT: Tea Tree oil

T: Peppermint oil NT: not tested

H: Manuka honey24+

3.3.6 The antibacterial activity of essential oils after being autoclaved

As the results in Figures 3.17, 3.18 and 3.19 show, there was generally not a statistically significant difference between autoclaved and non-autoclaved activities for essential oils against the tested bacteria. However, autoclaved Kanuka oil and Eucalyptus oil showed statistically significant difference between both activities against Gram-positive bacteria. Most results showed a slight decrease in antibacterial activity after autoclaving against all tested bacteria (Figs. 3.17, 3.18 and 3.19), except Tea Tree oil, which showed essentially the same effects against both Gram-positive bacteria (Figs. 3.17,3.18).

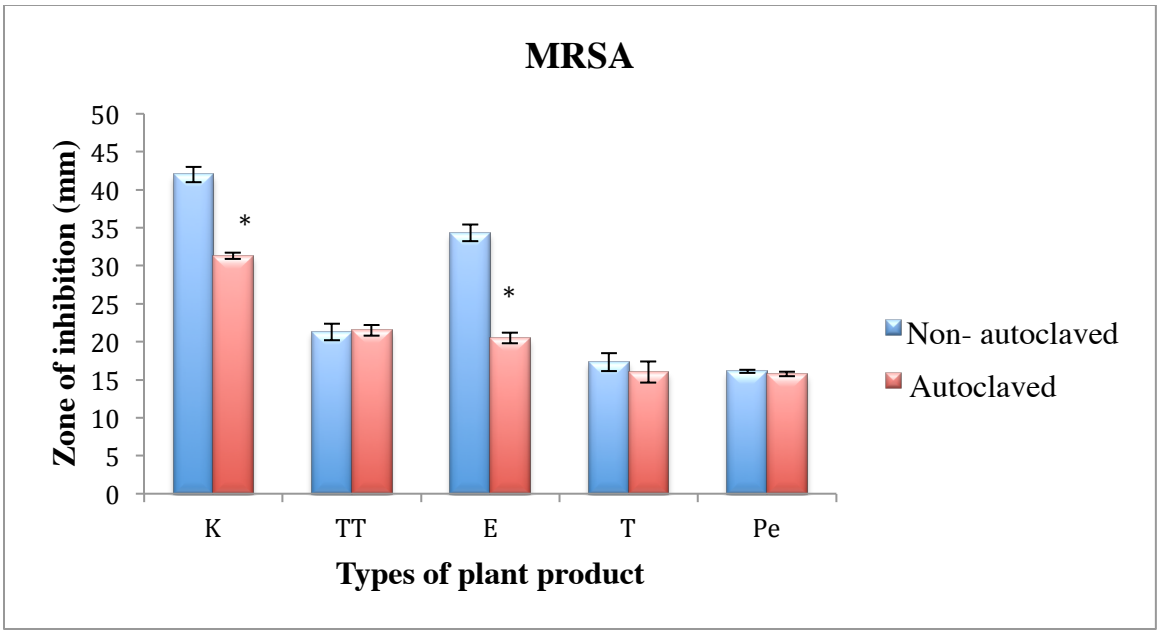

Figure 3.17 The antibacterial activity of autoclaved essential oils and non-autoclaved essential oils against MRSA determined by the well agar diffusion method. The values are means of triplicates ± Standard Deviation (including the well (8.0mm)).

* Statistically significant differences at $P \leq 0.05$.

Kanuka oil= (K)

Peru balsam oil= (Pe)

Eucalyptus oil= (E)

Tea Tree oil= (TT)

Peppermint oil= (T)

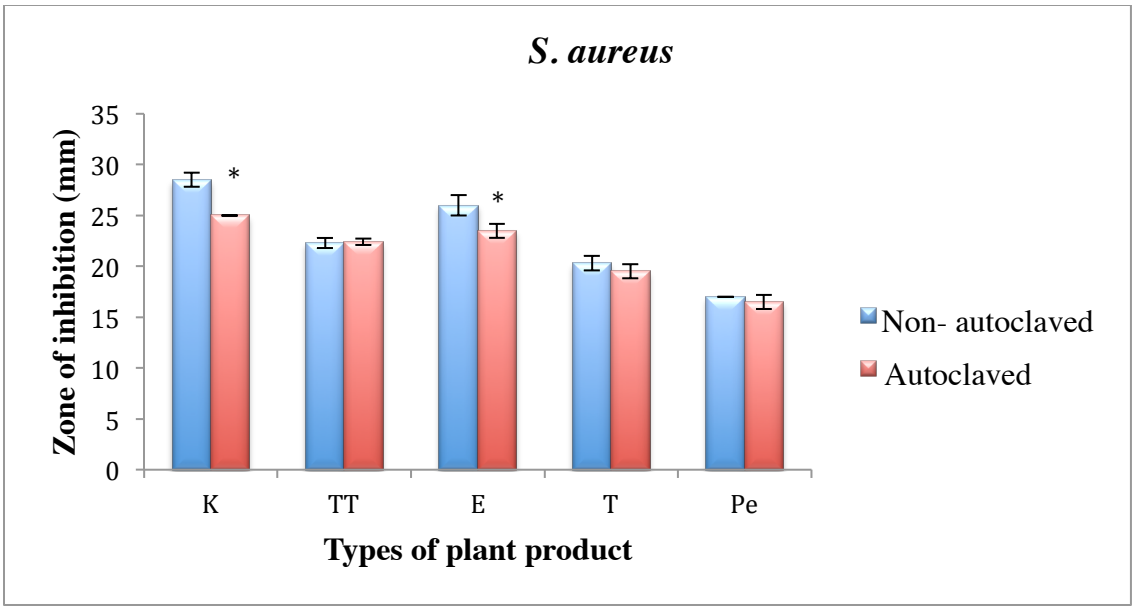

Figure 3.18 The antibacterial activity of autoclaved essential oils and non-autoclaved essential oils against *S. aureus* determined by the well agar diffusion method. The values are means of triplicates ± Standard Deviation (including the well (8.0mm)).

* Statistically significant differences at $P \leq 0.05$.

Kanuka oil= (K)

Peru balsam oil= (Pe)

Eucalyptus oil= (E)

Tea Tree oil= (TT)

Peppermint oil= (T)

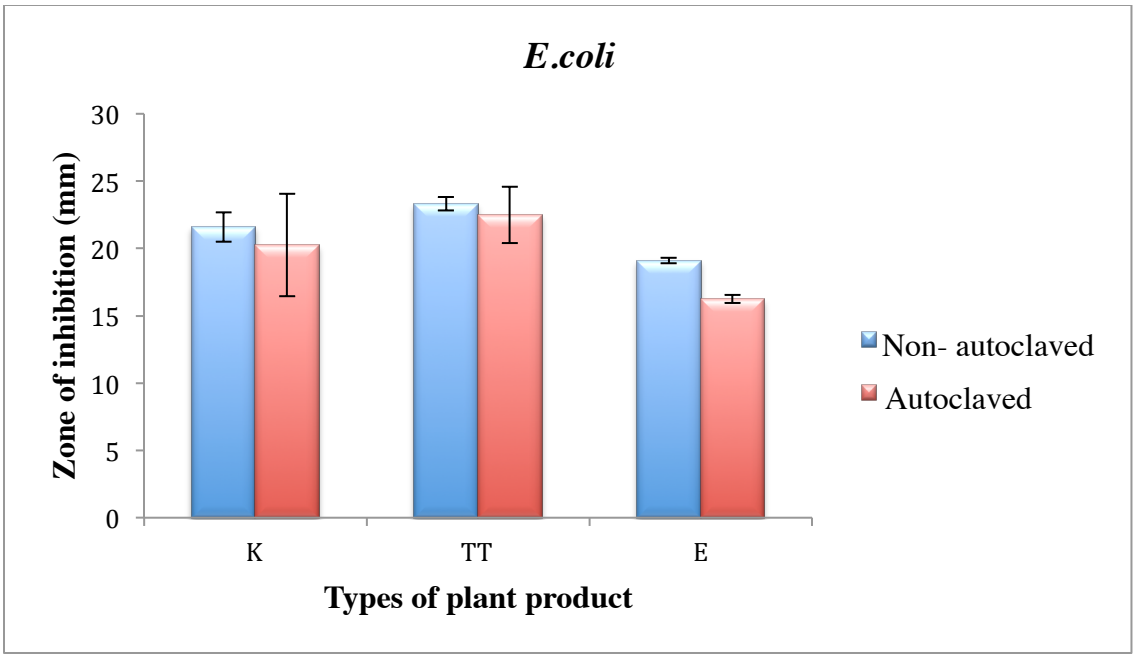

Figure 3.19 The antibacterial activity of autoclaved essential oils and non-autoclaved essential oils against *E.coli* determined by the well agar diffusion method. The values are means of triplicates ± Standard Deviation (including the well (8.0mm)).

There is no a statistically significant differences between the input groups $P \geq 0.05$.

Kanuka oil= (K)

Eucalyptus oil= (E)

Tea Tree oil= (TT)

3.4 Discussion

The results show that only five of the ten essential oils exhibited antimicrobial effects. The *in vitro* antimicrobial activities of these EOs were checked against Gram-negative bacteria, Gram-positive bacteria including methicillin-resistant *S. aureus* (MRSA) and the yeast species as indicated by the existence or non-existence of inhibition zones (i.e. the well agar diffusion assay), and by assessing MIC and MFC or MBC values. Generally, an increase in the concentration of the essential oils led to an increase in inhibition, as showed by the diameter of the inhibition zone. This result is similar to those found by Ghalem and Mohamed (2008) who reported that the bacterial growth rate decreased when the concentration of the essential oil was increased. Additionally, the larger inhibition zones were generally associated with lower MICs and this result agrees with those reported by Bassolé *et al.* (2010) who found that the biggest inhibition zones were associated with the lowest MIC values. In a few cases, the opposite of these findings was seen, a result supported by Sibanda and Okoh (2007) and Sun *et al.* (2014) who suggested that the efflux system can lead to a rise in the MIC of the drug, which is more than predicted, by reducing the intracellular concentration of the antimicrobial agent. TTO recorded the lowest MIC values (0.8%v/v) for both candida and the MFC

values are (0.8%v/v) for *Candida inconspicua* and (1.3%v/v) for *Candida rugosa*; this agrees with the result achieved by Carson *et al.* (2006) who mentioned that the MFCs range between 0.12 - 2% for yeasts generally. The diversity of the antimicrobial effects of the EOs could be attributed to many factors that produce varying effects on oil-composition; these include harvesting seasons (Burt, 2004), age and reproductive stage (Bakkali *et al*., 2008) and environmental factors such as temperature and rainfall (Grulova *et al*., 2015; Abdossi *et al*., 2015). For example, higher evaporation of volatile constituents of the essential oil is caused by higher temperatures (Grulova *et al*., 2015). Bakkali *et al.* (2008) concluded that in order to get essential oils with steady compositions, the extraction must be done under the same conditions including the same part of the plant, soil, climate and season. Moreover, the study by Demuner *et al.* (2011) showed that the harvest season contributed to the levels of major components of the essential oil of *Leptospermum madidum* subsp. *sativum* (Myrtaceae) grown in Brazil. Generally, the results showed that Gram-negative bacteria are more resistant to essential oils. This corresponds to the results reported by Palaniappan and Holley (2010) and Burt (2004) who pointed out that Gram-positive bacteria tend to be more susceptible to essential oils than Gram-negative, a finding that could be related to the different cell wall, and the

enzymes which are located in the peri-plasmic space, that make access to the bacterial cell more difficult (Nadjib *et al*., 2014). In addition, different antimicrobial activities of EOs were recorded ranging from strong to moderate to weak. These results are similar to those found by Chao *et al.* (2008) and Bassolé *et al.* (2010) who stated that the essential oils have different chemical components, which could be responsible for the diversity of their antimicrobial activities. In some results, the essential oils showed no observable zones of inhibition against organisms. This finding is in agreement with the work of Chao *et al.* (2008) who attributed the lack of antimicrobial activity of some essential oils against MRSA to these reasons: either the semi-solid surface of the media used in the disc diffusion method leads to difficulty in spreading the oils chemical components upon this surface, making difficult contact with MRSA; or possibly, because essential oils are hydrophobic and they cannot spread optimally under this aqueous environment; or finally, it may be due to the volatility and miscibility of essential oils. In this study, Eucalyptus oil, Kanuka oil and Tea Tree oil showed antimicrobial activity against *Staphylococcus aureus*, MRSA and *Escherichia coli* as well as the antifungal activity against *Candida inconspicua* and *Candida rugosa*. This result supports the findings of Chen *et al.* (2016) who found that *K. ericoides* significantly inhibited eight

microorganisms including (*Malassezia furfur, Trichosporon mucoides, Candida tropicalis, Candida albicans, Escherichia coli, Streptococcus sobrinus, Streptococcus mutans* and *Staphylococcus aureus*); and the same authors suggested that Kanuka oil could be a useful candidate for use in pharmaceutical antibiotics. A study by Ghalem and Mohamed (2008) demonstrated that the eucalyptus species exhibit antibacterial activity against both Gram-positive and Gram-negative resistant bacteria. On the other hand, these results are in disagreement with findings by Wyatt *et al.* (2005) who mentioned that "Kanuka oil cannot be recommended for antimicrobial purposes". Another study by Khan *et al.* (2009) noted that *Eucalyptus globules* had an effect on Gram-positive bacteria only. Additionally, Peru balsam oil and Peppermint oil were effective against Gram-positive bacteria, including MRSA and not effective against *Escherichia coli*. This result is comparable to studies by Demuner *et al.* (2011) who reported that the essential oils of *Leptospermum petersonii* (Myrtaceae) showed antimicrobial activity against Gram-positive bacteria and no antimicrobial activity against *Escherichia coli*. In some cases, the combination of essential oils with Manuka honey has shown a greater inhibitory effect in comparison to the oils acting individually. This result is similar to the work carried out by Van Vuuren *et al.* (2014) who found that

the combination of essential oils is often a way to increase their efficacy. In addition, essential oils were shown to be heat resistant or thermostable since the antimicrobial activities were not affected after autoclaving (Helal *et al*., 2006; Khafagi *et al*., 2000). Their findings disagree with this study, which showed that autoclaved Kanuka oil and Eucalyptus oil showed a decrease in antibacterial activity against Gram-positive bacteria.

In conclusions, essential oils have a marked potential for use as antimicrobial compounds against a wide range of microorganisms. Therefore these extracts should be considered for use in the treatment of infectious diseases, which result from the activity of antibiotic resistant microbes, in particular, MRSA.

Chapter Four: Comparing Between The Antimicrobial Activity of Herbal Extracts Alone and in Various Combinations

4.1 Introduction

4.1.1 Essential oils and plant extracts for use in combination

Several studies have reported the antimicrobial properties of plant extracts, and essential oils as secondary metabolites derived from plants (Aiyegoro *et al*., 2009; Mikulášová *et al*., 2016), as terpenoids and terpenes (Darwish *et al*., 2002). For many years, healers have used combinations of plants to treat infections (Aiyegoro and Okoh, 2009) and their use has been documented over than 2,000 years ago in Chinese medicine (Che *et al*., 2013). Plant extracts inhibit bacteria by different mechanisms due to their many different phytochemicals and the effect of these combinations among herbs can be complicated, due to a variety of different interactions that can occur between the individual constituents (Che *et al*., 2013). So, a synergistic or an additive effect could result from this double effect of two agents on multi-target sites of the bacteria (Adwan and Mhanna, 2008) with the additional benefit of lowering the necessary dose and cost (Krychowiak *et al*., 2014). Also, Aiyegoro *et al.* (2009) illustrated that the aims of the combination therapy are achieve synergy, decrease toxicity, and reduce or postpone the bacterial resistance. As a rule, essential oils are generally used in monotherapy, but they can also be used in combination therapy (De Rapper *et al*., 2013). De Rapper *et al.* (2013) pointed out that up to 600

potential essential oil combinations have been identified as antimicrobial agents. The interactions between the main components in essential oils appear to be associated to the antimicrobial activities (Bassolé *et al*., 2010). Aiyegoro and Okoh (2009) found that a synergistic effect could result from the increase of biological activity by phenolic compounds such as flavonoids. Furthermore, Dawoud *et al.* (2013) showed that the combination of *Rehum palmatum*, *Cassia angustifolia* and *Glycyrrhiza glabra* led to an increase in antibacterial activity. Clinically, synergistic actions between Chinese herbs used to cure eczema were reported (Aiyegoro and Okoh, 2009).

The aim of the experimental work described in this Chapter was to investigate the possible synergistic effect of the combination of plant extracts against microorganisms.

4.2 Materials and methods

4.2.1 Plant extract sample preparation

Five essential oil samples (Kanuka oil, Peru balsam oil, Eucalyptus oil, Tea Tree oil and Peppermint oil) and four herbal extracts (Grapefruit seed extract, Goldenseal extract, Noni juice and Derum) were generally used undiluted, although (50%v/v) sample concentrations were also prepared.

4.2.2 Test organisms

The following test microorganisms were used: *Staphylococcus aureus*, Methicillin-Resistant *S. aureus* MRSA, *Escherichia coli*, *Candida rugosa* and *Candida inconspicua*.

4.2.3 Inoculum preparation and turbidity standard

The inocula of susceptibility tests were adjusted to $1.5x10^8$ CFU/ml which reference to the (0.5 McFarland standards). Two to five isolated colonies from pure microbial culture were taken using a sterile loop and then inoculated into a tube containing distilled, sterile water and mixed by using a vortex until the mixture became homogeneous. The turbidity was measured by a spectrophotometer at 600 nm for bacteria, and at 580 nm for yeast. These suspensions were used within 30 minutes of preparation (Andrews, 2001).

4.2.4 Evaluation of the synergistic effect of combining two plant extracts

In order to study the synergistic effect of combining two plant extracts by agar well diffusion assay, the plates were prepared using 20 ml of sterile media. A total of 0.1 ml of bacterial suspension adjusted to 0.5 McFarland turbidity standard was poured onto each plate containing Muller-Hinton agar. A sterile glass spreader was used to distribute the inoculum on the surface of agar. All plates were allowed to dry for 30 minutes. Wells, 8.0 mm in diameter, were cut from the culture media using a sterile metal cylinder and then filled with a volume of 0.1 ml (when tested individually), which used as control, and 0.05 ml: 0.05 ml (when tested in combination); each well received 0.1ml. After a 30 min pre-diffusion time interval, the Petri dishes were subsequently incubated at 37°C for 24 hours and the inhibition zone around each well was measured in mm, including the well (8.0mm). The result was then recorded. The antifungal activity of combining two plant extracts was determined with the same process using *Candida rugosa* and *Candida inconspicua*, which was adjusted to 0.5 McFarland turbidity standards, and 0.1 ml suspension was spread onto the surface of Saboraud Dextrose agar.

4.3 Results

4.3.1 Evaluation of the synergistic effect of combining two plant extracts

The most active plant extracts (Kanuka oil, Eucalyptus oil, Grapefruit seed extract and Goldenseal extract (DL)) were selected for additional study using the agar well diffusion method.

4.3.1.1 Kanuka oil

Table 4.1 shows the antimicrobial activity of Kanuka oil, evaluated in combination with four essential oils (Eucalyptus oil, Peru balsam oil, Tea Tree oil and Peppermint oil) to determine interactive properties by using the agar well diffusion assay. Generally, all essential oils in combination with Kanuka oil showed antagonistic results against the growth of the microorganisms (Table 4.1). However, Kanuka oil raised the efficacy of all oils when combined with them and showed a greater inhibition zone than the oil independently, with the exception of Tea Tree oil and Kanuka oil (19.9±0.6) and Tea Tree oil alone (23.6±0.5) against *S. aureus* (Table 4.1). Furthermore, the effect of Kanuka oil in combination was decreased when compared with the effect of Kanuka oil alone against Gram-positive bacteria, except in the case of Eucalyptus oil with Kanuka oil against *S.*

aureus, as well as Tea Tree oil with Kanuka oil against *E.coli* (Table 4.1). Moreover, the inhibition zone of all combinations, for all oils including Kanuka oil, increased in comparison with each oil individually against both *Candida* species respectively (Table 4.1).

4.3.1.2 Eucalyptus oil

The antimicrobial activity of Eucalyptus oil alone, and in combination with four essential oils (Kanuka oil, Peru balsam oil, Tea Tree oil and Peppermint oil), was tested by using the agar well diffusion assay as shown in Table 4.2. All results showed an antagonistic effect against all tested microorganisms (Table 4.2). Otherwise, the efficacy of the combinations was increased when combined with each of the oils on its own and showed a greater inhibition zone than individually against all microbial materials tested, except the combination of Eucalyptus oil with Kanuka oil against both MRSA and *E.coli* (Table 4.2).

4.3.1.3 Grapefruit seed extract

Table 4.3 shows the antimicrobial activity of Grapefruit seed extract in combination with five essential oils (Kanuka oil, Eucalyptus oil, Peru balsam oil, Tea Tree oil and Peppermint oil) and four herbal extracts (Goldenseal extract D and DL, Derum and Noni juice). In this study, no

synergy was observed for any of the extract combinations (Table 4.3). However, the diameter of the zone of inhibition of the extracts in combination with Grapefruit seed extract was greater than the effect of the extract acting independently, with the exception of the combination of Grapefruit seed extract with Kanuka oil, Eucalyptus oil and Derum against *E.coli* (Table 4.3). Other exceptions were Goldenseal extract D and DL, Derum and Tea Tree oil against *S. aureus* (Table 4.3). Also in the case of MRSA, the inhibition zone of Goldenseal extract DL and Derum was negated when they were combined with Grapefruit seed extract and even the effect of Grapefruit seed extract was negated, as it was when Grapefruit seed extract was combined with Goldenseal extract D against *S. aureus* and MRSA (Table 4.3). In addition, the effect of Grapefruit seed extract in combination with Peru balsam oil or Noni juice was decreased when compared with the effect of Grapefruit seed extract alone against MRSA (Table 4.3). Interestingly, Grapefruit seed extract alone had no effect against *E.coli* but the effect was increased for both extracts when combined with Noni juice or Tea Tree oil (Table 4.3); as did Grapefruit seed extract and Noni juice against *S. aureus*.

4.3.1.4 Goldenseal extract (DL)

The synergistic interactions between Goldenseal extract (DL) alone and in

combination with five essential oils (Kanuka oil, Eucalyptus oil, Peru balsam oil, Tea Tree oil and Peppermint oil), also with four other herbal extracts (Goldenseal extract D, Derum, Grapefruit seed extract and Noni juice) were studied as shown in Table 4.4. Antagonistic effects were observed for almost all combinations, while the interactions between Goldenseal extract (DL) and Eucalyptus oil were synergistic against *Candida rugosa* and *Candida inconspicua,* as were Goldenseal extract (DL) and Peppermint oil against *Candida inconspicua* (Table 4.4). Furthermore, the additive effect was observed on a single occasion when Goldenseal extract (DL) and Kanuka oil were combined against *Candida rugosa* (Table 4.4). Otherwise, the diameter of the zone of inhibition of extracts in combination with Goldenseal extract (DL) was greater than the effect of the extract acting independently in almost all results (Table 4.4). However, in some cases, combinations exhibited either a decreased effect of Goldenseal extract (DL) when combined with Grapefruit seed extract, Derum and Peru balsam oil than when used alone against *S. aureus,* as did Goldenseal extract (D) and Derum against MRSA, or negated the effect such as the combination of Derum or Noni juice with Goldenseal extract (DL) against *Candida rugosa* (Table 4.4). Also, the effect of both extracts in combination was negated in comparison to the extracts acting individually,

for instance, combinations of Goldenseal extract (DL) with Grapefruit seed extract against MRSA or with Derum against *Candida inconspicua* (Table 4.4).

Table 4.1 Effect of combinations of Kanuka oil and other essential oils against microbial strains determined by the agar diffusion assay. Means of triplicates ± Standard Deviation (including the well (8.0 mm)).

Bacteria	**Essential Oils Combination**								
	K	Pe	Pe+k	E	E+K	T	T+K	TT	TT+K
S. aureus	20.5±0.5	14 ±1	16.8±0.2	15.9±0.8	21±0.7	14 ±1	18±1	23.6±0.5	19.9±0.6
E.coli	16.3±1.5	8±0	NT	15.5±0.5	16±1	8±0	NT	18±1	25.3±0.3
MRSA	25.3±0.2	12.6±1.1	19.6±1.5	18.3±1.5	21±1.3	15.3±0.5	17.5±1.3	13.8±0.7	15.6±0.7
Candida inconspicua	12.3±0.5	17.6±1.5	21±1	15.3±0.5	18.8±1	16.6±1.1	24±1	24.6±0.5	29.1±1.7
Candida rugosa	13.3±1.1	19.3±1.1	19.5±0.7	15±1	21.7±1.7	21.6±1.1	21.7±1	27±1.4	35.2±0.3

K: Kanuka oil Pe: Peru balsam oil

E: Eucalyptus oil TT: Tea Tree oil

T: Peppermint oil NT: not tested

Table 4.2 Effect of combinations of Eucalyptus oil and other essential oils against microbial strains determined by the agar diffusion assay. Means of triplicates ± Standard Deviation (including the well (8.0 mm)).

Bacteria	**Essential Oils Combination**								
	E	K	E+K	Pe	E+Pe	T	E+T	TT	E+TT
S. aureus	15.9±0.8	20.5±0.5	21±0.7	14 ±1	24.1±0.2	14 ±1	23.5±0.5	23.6±0.5	28.6±1.1
E.coli	15.5±0.5	16.3±1.5	16±1	8±0	NT	8±0	NT	18±1	21.5±0.9
MRSA	18.3±1.5	25.3±0.2	21±1.3	12.6±1.1	26.1±0.2	15.3±0.5	21.6±0.2	13.8±0.7	21.5±0.5
Candida inconspicua	15.3±0.5	12.3±0.5	18.8±1	17.6±1.5	22.6±0.5	16.6±1.1	23.6±0.5	24.6±0.5	36.5±0
Candida rugosa	15±1	13.3±1.1	21.7±1.7	19.3±1.1	23.1±0.2	21.6±1.1	21.8±0.2	27±1.4	27.3±0.5

K: Kanuka oil Pe: Peru balsam oil

E: Eucalyptus oil TT: Tea Tree oil

T: Peppermint oil NT: not tested

Table 4.3 Effect of combinations of Grapefruit seed extract and other plant products against microbial strains determined by the agar diffusion. Means of triplicates ± Standard Deviation (including the well (8.0 mm)).

Plant products Individual and in combination	**Microbial Strains**		
	MRSA	***S. aureus***	***E.coli***
GSE	16±0	14.3±1.1	8±0
K	25.3±0.2	20.5±0.5	16.3±1.5
GSE+K	26.5±0.8	26.6±1.1	15.8±0.5
E	18.3±1.5	15.9±0.8	15.5±0.5
GES+E	20.6±0.5	22.6±1.5	13.6±0.2
T	15.3±0.5	14±1	8±0
GSE+T	18.5±0.5	20.1±0.2	NT
TT	13.8±0.7	23.6±0.5	18±1
GSE+TT	19±0.8	18.1±0.2	20±0.5
Pe	12.6±1.1	14±1	8±0
GSE+Pe	15.3±0.5	17.3±0.5	NT
D	8±0	10.6±0.5	8±0
GSE+D	8±0	8±0	NT
Dl	19.6±0.5	22.5±0.5	8±0
GSE+Dl	8±0	20.6±0.7	NT
Dr	15.3±0.2	16.6±0.5	11±0
GSE+ Dr	8±0	13.6±0.5	10.8±1.6
N	8±0	8±0	8±0
GSE+N	13.3±0.5	15.33±0.5	11.6±0.5

K: Kanuka oil Pe: Peru balsam oil E: Eucalyptus oil

TT: Tea Tree oil T: Peppermint oil NT: not tested

GSE: Grapefruit seed extract D: Goldenseal extract N: Noni juice

DL: Goldenseal extract Dr: Derum

Table 4.4 Effect of combinations of Goldenseal extract and other plant products against microbial strains determined by the agar diffusion. Means of triplicates ± Standard Deviation (including the well (8.0 mm)).

Plant products Individual and in combination	**Microbial Strains**			
	MRSA	***S. aureus***	***Candida inconspicua***	***Candida rugosa***
Dl	19.6±0.5	22.5±0.5	14.3±0.5	15.3±2
K	25.3±0.2	20.5±0.5	12.3±0.5	13.3±1.1
Dl+K	27.5±0.8	32.6±0.5	25.1±0.5	28.6±0.2
E	18.3±1.5	15.9±0.8	15.3±0.5	15±1
Dl +E	26.6±0.5	29.5±0.5	29.8±0.2	32.5±0.8
T	15.3±0.5	14±1	16.6±1.1	21.6±1.1
Dl +T	29.8±0.7	28.1±0.5	37.1±0.2	32.3±0.5
TT	13.8±0.7	23.6±0.5	24.6±0.5	27±1.4
Dl +TT	25.1±0.5	27.6±0.2	35.6±0.2	33.5±0.5
Pe	12.6±1.1	14±1	17.6±1.5	19.3±1.1
Dl +Pe	20.6±0.5	22.3±0.5	21.6±0.2	23.1±0.2
D	8±0	10.6±0.5	8±0	8±0
Dl +D	17.5±0.5	22.6±0.5	NT	NT
GSE	16±0	14.3±1.1	8±0	8±0
Dl +GSE	8±0	20.6±0.7	NT	NT
Dr	15.3±0.2	16.6±0.5	10±0	8±0
Dl +Dr	18.8±0.7	19.1±1	8±0	8±0
N	8±0	8±0	8±0	8±0
Dl+N	19.6±0.2	23.3±0.5	NT	8±0

K: Kanuka oil Pe: Peru balsam oil E: Eucalyptus oil

TT: Tea Tree oil T: Peppermint oil NT: not tested

GSE: Grapefruit seed extract D: Goldenseal extract N: Noni juice

DL: Goldenseal extract Dr: Derum

4.4 Discussion

Generally, the combination of essential oils and herbal extracts with each other showed a greater inhibitory effect in comparison to the oils or herbal extracts when used individually. This is not surprising, since each plant extract has many components which can act positively to inhibit pathogens, a result which is in agreement with Che *et al*. (2013) who reported that plant extracts inhibit bacteria by different mechanisms due to their many different phytochemicals and the effect of these combinations among herbs can be complicated, due to several different interactions that can happen between the individual constituents. Maddocks-Jennings *et al.* (2005) showed that a combination of Manuka oil and Kanuka oil were more effective. As the results shown here illustrate, the combinations between Goldenseal extract (DL) and Eucalyptus oil produced a synergistic effect against *Candida rugosa* and *Candida inconspicua;* this synergistic effect was also seen for combined Goldenseal extract (DL) and Peppermint oil against *Candida inconspicua.* Furthermore, the additive effect was shown for combined Goldenseal extract (DL) and Kanuka oil against *Candida rugosa*. This result is agreement with the study carried out by Van Vuuren *et al.* (2014) who found that the combination of essential oils often increases their efficacy. Chao *et al.* (2008) suggested that microbes cannot

develop resistance to all active components in two oils at the same time and Chao *et al.* (2008) suggested that the possible interaction between active components as they arise in plants provides these plants with their antimicrobial activity, such as Tea Tree oil which has over 100 components, and many of them exhibit natural antimicrobial character for instance terpinene and 1,8-cineole. Although 1,8-cineole has a limited antimicrobial activity, it is effective by enhancing terpinene, possibly by its ability to permeabilize bacterial membranes, which then allows more terpinene to enter and destroy the bacterial cell (Chao *et al.,* 2008). This suggestion which showed that one component was involved in enhancing the efficacy of the others could explain the result of the synergistic effect between two agents reported here; when two plant extracts are combined, one of them showed no effect, for example the combination of Noni juice with Goldenseal extract (DL) against *S. aureus.* Surprisingly, in some rare cases, plant products alone succeeded in inhibiting pathogens, while a combination of antimicrobial agents blocked or decreased the effectiveness of one or both agents; lack of antimicrobial activities on some combinations is undesirable and more research is required so this effect can be obviated during practical applications.

In conclusion, the synergistic interaction between the combinations of two

plant products against microorganisms may be used to provide potential novel approaches to the treatment of infectious diseases. However, essential oils or some plant extracts should be used with care when combined with other plant products as one of them could inhibit the positive effect of the others.

Chapter Five: The Effects of Combining Herbal Extracts and Common Antibiotics on Pathogens

5.1 Introduction

5.1.1 Combined antibiotic therapy

Bacterial pathogens are increasingly becoming resistant to many clinically available antibiotics. In order to control antibiotic resistance, there is a critical and urgent need for alternatives or for the development of new antibiotics. Today, there is a considerable trend towards controlling resistant pathogens by the use of current antibiotics combined with natural antimicrobial agents in combinations against bacteria (Tran *et al*., 2012). Rodrigues *et al.* (2009) termed the use of herbals and antibiotics in a multi targeted strategy, a 'herbal shotgun'' and referred to ''synergistic multi-target effects'' and noted that mono or multi extract combinations can act not only on one target, but diverse ones. Adwan and Mhanna (2008) stated that combined antibiotic therapy might be used to treat bacterial infections through postponing bacterial resistance and also creating effective synergistic interaction between conventional antibiotics and bioactive plant extracts. In some cases, antibiotics alone do not succeed in inhibiting pathogens while a combination of antimicrobial agents often result in a synergistic effect (Chanda and Rakholiya, 2011). Bacteria have a mechanism (efflux pumps) that is responsible for an important level of resistance to antibiotics since bacteria use this mechanism to pump out

antibiotics and chemicals (Tran *et al*., 2012; Mikulášová *et al*., 2016). Aiyegoro and Okoh (2009) reported that some plant derived compounds inhibited multidrug resistance efflux systems in bacteria and as a result, antibiotics may target bacteria more effectively, depending on antibiotic active components such as the aminoglycoside antibiotics (e.g. gentamicin) which inhibits amino acid polymerisation, also vancomycin, which inhibits bacterial cell wall synthesis and tetracycline that inhibits ribosomal protein synthesis (Dawoud *et al*., 2013). The combination of antibiotics and plant extracts can also minimize toxic effects as well as decreasing the required dose of drugs (Aiyegoro and Okoh, 2009; Aiyegoro *et al*., 2009). Rosato *et al.* (2007) reported that the essential oil of *Pelargonium graveolens* decreased the minimum efficient dosage of norfloxacin against *Staphylococcus aureus, Bacillus subtilis, Bacillus cereus* and *Escherichia coli*. However, some plants do not exhibit any antimicrobial properties if used alone, but when they are combined with antibiotics they can increase drug-efficiency (Chanda and Rakholiya, 2011; Aiyegoro and Okoh, 2009; Mikulášová *et al*., 2016). Adwan and Mhanna (2008) commented on the fact that the efficacy of antimicrobial agents could likely be enhanced by combining them with crude plant extracts. Several *in vitro* studies have reported synergistic effects of essential oils with antibiotics, which can be

used to minimize microbial resistance to traditional drugs (Van Vuuren *et al*., 2009).

The aim of the experimental work described in this chapter was to investigate if the combination of different plant extracts with selected antibiotics improved the efficacy of antibiotics through increasing the susceptibility of tested bacteria when compared with the action of these antibiotics alone.

5.2 Materials and methods

5.2.1 Plant extracts sample preparation

Five herbal extracts (Grapefruit seed extract, Goldenseal extract (DL), Goldenseal extract (D), Derum and Noni juice) and five essential oil (Kanuka oil, Eucalyptus oil, Peru balsam oil, Tea Tree oil and Peppermint oil) samples were generally used undiluted.

5.2.2 Test organisms

The following test microorganisms were used: *Staphylococcus aureus*, Methicillin-Resistant *S. aureus* MRSA and *Escherichia coli*.

5.2.3 Inoculum preparation and turbidity standard

The inocula of susceptibility tests were adjusted to $1.5x10^8$ CFU/ml which reference to the (0.5 McFarland standards). Two to five isolated colonies from pure microbial culture were taken using a sterile loop and inoculated into a tube containing distilled, sterile water and mixed by using a vortex until the mixture became homogeneous. The turbidity was measured by a spectrophotometer at 600 nm. These suspensions were used within 30 minutes of preparation (Andrews, 2001).

5.2.4 Evaluation of the effect of combining antibiotics and plant extracts

In order to determine the synergistic interaction between six selected antibiotic discs, including vancomycin 30 µg, ampicillin 10 µg, erythromycin 15 µg, chloramphenicol 30 µg, gentamicin 10 µg and tetracycline 30 µg, (purchased from Fisher Scientific (Oxoid™), with plant extract samples, the agar disc diffusion method was used with slight modification; the plates being prepared using 20 ml of sterile media and inoculated with $1.5x10^8$ CFU/ml with reference to the 0.5 McFarland turbidity standard. All plates were allowed to dry for 30 minutes. Antibiotic discs were placed on the surface of Muller Hilton agar. 100µl of each sample was poured on top of all antibiotic discs. Antibiotic discs alone were used as control. After a 30 min pre-diffusion time interval, the Petri dishes were incubated at 37°C for 24 hours and the inhibition zone around each disc was measured in mm, including disc (6mm); the result was then recorded.

5.2.5 Statistical Analysis

All observations were presented as Mean ± SD (Standard Deviation). The data were analysed by IBM Corp© 24.0. One way ANOVA was performed to compare if there was a significance difference of the inhibition zone

values measured between the different combination of antibiotics and plant products against the test organisms. $P \leq 0.05$ was considered as statistically significant. Tukey-Post-Hoc test confirmed the pairwise comparisons.

5.3 Results

5.3.1 Evaluation of the effect of combining antibiotics with plant extracts

The combination of plant extracts and cell wall biosynthesis antibiotics, β-lactam antibiotics and glycopeptides agents (ampicillin and vancomycin), and protein synthesis-inhibiting antibiotics (tetracycline, gentamicin, erythromycin and chloramphenicol) was studied both individually and in combinations. Generally, plant extracts improved the efficacy of almost all antibiotics and the interaction between plant extracts and antibiotics had more inhibitory effects than antibiotics alone (Tables 5.1-5.4). However, a combination of erythromycin 15 µg and Grapefruit seed extract (Tables 5.1 and 5.2) showed a decrease in the inhibitory effect against all Gram-positive bacteria as well as the combination of erythromycin 15 µg and Noni juice against *S. aureus* (Table 5.2). In addition, the combination of Peru balsam oil and two antibiotics (tetracycline 10 µg and chloramphenicol 30 µg) led to a notable decrease in the inhibitory effect against *S. aureus*, (Table 5.2) as did gentamicin 30 µg and Derum against *S. aureus* (Table 5.2).

Generally, *E.coli* exhibited the most resistance, with the size of the

inhibition zones increasing for more than half the combination results while the remainder showed a decrease or no inhibitions (Table 5.3). Specifically, the combinations of plant extracts, including Kanuka oil with antibiotics containing ampicillin 10 µg, chloramphenicol 30 µg, vancomycin 30 µg and erythromycin 15 µg, and Noni juice with antibiotics containing ampicillin 10 µg, chloramphenicol 30 µg and erythromycin 15 µg were ineffective and also removed the individual effect of plant extracts, as did the combinations of Derum and ampicillin 10 µg and erythromycin 15 µg (Table 5.3). Also, the combination of Grapefruit seed extract and gentamicin 30 µg (Table 5.3) was ineffective and had the same inhibition zone as antibiotic alone, as demonstrated in Table 5.4.

Table 5.1 Effect of the combination of antibiotics and plant extracts against MRSA determined by use of the disc diffusion assay. Means of triplicates ± Standard Deviation (including the size of the disc (6.0mm)).

	Inhibition zone (mm)±SD					
	TE	**V**	**ER**	**A**	**CL**	**GE**
Kanuka oil	66.5±0.5	70.6±0.7	62.6±0.5	61.1±1	67.8±1.6	61.8±1.4
Eucalyptus oil	33.6±0.7	26.8±1	40±0.5	39.5±0.5	36.1±1.8	28.5±1.8
Peru balsam oil	22.6±0.5	23.6±1	24.5±1.3**	28.8±1.2	22.6±1**	23.5±1.3
Peppermint oil	67.2±1.9	62.8±1	66±1.5	73.6±1.3	56±1.4	63.3±1.5
Tea Tree oil	48.6±0.5	44±1.3	60.5±0.5	46.3±0.5	50.1±1.2	43.5±1.8
Goldenseal	32.3±0.7	34±1.7	35.5±0.8	40.1±2	33.6±1	40.8±0.7
Noni juice	30.1±0.7	19.6±0.2**	24.1±1**	28.1±1	23.5±0**	19.1±0.2**
Derum	29.6±0.5	20.6±0.5	31.8±0.2	24±0.8	25.6±0.2**	20±0.5
Goldenseal (D)	26.3±0.5	22.8±0.2	29.8±0.5	25.6±0.2	25.6±0.7**	20.1±0.5
Grapefruit seed extract	29±0.2	20.5±1.3	16±1.3	30.3±2	25±2**	17.6±1.8**
Control	20.3±0.5*	15±0	23.6±0.2	22.5±0.5*	22.5±0.8	17±0

*Statistically significant difference between input groups ($p \leq 0.05$).

** No statistically significant difference between input groups ($p \leq 0.05$).

V: vancomycin

ER: erythromycin

A: ampicillin

CL: chloramphenicol

GE: gentamicin

TE: tetracycline

Control: antibiotic alone

Table 5.2 Effect of the combination of antibiotics and plant extracts against *S. aureus* determined by use of the disc diffusion assay. Means of triplicates ± Standard Deviation (including the size of the disc (6.0mm)).

	Inhibition zone (mm)±SD					
	TE	**V**	**ER**	**A**	**CL**	**GE**
Kanuka oil	67.3±0.2	65.3±1.5	67.3±1.5	63.5±1.3	61.7±0.9	43.3±1.5
Eucalyptus oil	28.5±0.5	26.3±1.5	39.5±1.3	48.3±0.2	37.4±1	28.5±0.8
Peru balsam oil	22.8±0.2	23±1.3	29±1.7	39.1±1	23±1.8**	23.5±1.3**
Peppermint oil	59±1	49.8±1.3	69.3±1	62.5±1.5	56.6±0.7	62.2±1.5
Tea Tree oil	58.1±1	63.1±0.2	62±1	53.4±0.8	62.5±1.5	67.1±0.2
Goldenseal (DL)	36.6±0.5	39.8±1.2	38.3±1	44.6±1.5	42.3±1.2	43.6±0.2
Noni juice	30.3±0.5	20.3±0.5	22.6±0.5**	36.3±0.5	24.6±0.5**	23.6±0.5
Derum	29.3±0.2	22.3±0.2	34.8±0.7	35.1±0.7	27.5±0.5	20.3±0.5**
Goldenseal (D)	28±0.5	20.1±0.2	30.5±0.5	37.6±0.5	25±0.5**	24.3±0.2
Grapefruit seed extract	29.6±0.5	23.1±1	14.8±1.6	41.1±0.2	26±1**	24.1±0.7
Control	23.8±0.2*	15±0*	25.1±0.7	31.1±0.2*	24.5±0.5	20.6±0.5

*Statistically significant difference between input groups ($p \leq 0.05$).

** No statistically significant difference between input groups ($p \leq 0.05$).

V: vancomycin

ER: erythromycin

A: ampicillin

CL: chloramphenicol

GE: gentamicin

TE: tetracycline

Control: antibiotic alone

Table 5.3 Effect of the combination of antibiotics and plant extracts against *E.coli* determined by use of the disc diffusion assay. Means of triplicates ± Standard Deviation (including the size of the disc (6.0mm)).

	Inhibition zone (mm)±SD					
	TE	**V**	**ER**	**A**	**CL**	**GE**
Kanuka oil	20.3±0.5	6±0	6±0**	6±0**	6±0	21±1
Eucalyptus oil	24.3±0.5	16.6±0.5	24.6±1.1	20.6±2	21.5±1.3	28.8±1
Tea Tree oil	55.3±0.5	68.5±0.5	59±1	63.3±1.1	60±1	64.6±1.5
Noni juice	22.3±0.5	10±0	6±0**	6±0**	6±0**	23.6±1.1
Derum	23.3±0.5	10.3±0.5	6±0**	6±0**	10.6±0.5	18.6±0.5**
Grapefruit seed extract	23.6±0.5	19.3±1.5	17.3±0.5	20.5±1.8	15.5±0.5	17.6±1.5**
Control	18±0*	8.3±0.5*	6±0	6±0	6±0	17.6±0.5

*Statistically significant difference between input groups ($p \leq 0.05$).

** No statistically significant difference between input groups ($p \leq 0.05$).

V: vancomycin

ER: erythromycin

A: ampicillin

CL: chloramphenicol

GE: gentamicin

TE: tetracycline

Control: antibiotic alone

Table 5.4 Effect of antibiotics against bacteria determined by use of the disc diffusion assay. Means of triplicates ± Standard Deviation (including the size of the disc (6.0mm)).

Antibiotics	Inhibition zone (mm)±SD		
	MRSA	***S. aureus***	***E.coli***
Tetracycline	20.3±0.5	23.8±0.2	18±0
Vancomycin	15±0	15±0	8.3±0.5
Erythromycin	23.6±0.2	25.1±0.7	6±0
Ampicillin	22.5±0.5	31.1±0.2	6±0
Chloramphenicol	22.5±0.8	24.5±0.5	6±0
Gentamicin	17±0	20.6±0.5	17.6±0.5

5.4 Discussion

The new approaches to treatment of infectious diseases resulting from the use of multidrug-resistant microorganisms could likely be achieved by using a combination of essential oils and conventional antibiotics (Yap *et al.,* 2014). Generally, plant extracts improved the efficacy of almost all antibiotics and the interaction between plant extracts and antibiotics had more inhibitory effects than antibiotics alone. This result was supported by other results which indicated that the combinations of plant products with antibiotics dramatically enhanced the activity of some antibiotics even against drug-resistant strains of bacteria (Sibanda and Okoh, 2007), where the active efflux is a possible mechanism of resistance in almost all (Adwan *et al*., 2010). Aiyegoro and Okoh (2009) stated that some plant-derived compounds are responsible for inhibiting multi drug resistant (MDR) efflux systems in bacteria, while studies by Van Vuuren *et al.* (2009) and Esimone *et al.* (2006) show that essential oil components and antibiotics attack microbes at differing target sites, thereby improving antibiotic efficiency. This study is comparable to a number of *in vitro* studies by Chao *et al.* (2008), Sibanda and Okoh (2007), Yap *et al.* (2014), Nascimento *et al.* (2000) and Chanda and Rakholiya (2011) who also reported synergistic effects between plant extracts and antibiotics. Darwish *et al.* (2002) pointed

out that the combination of gentamicin and chloramphenicol and some Jordanian plant materials against *S. aureus* leads to improved efficacy of antibiotics, while Rodrigues *et al.* (2009) reported that the essential oil of *Croton zehntneri* leaves enhances gentamicin activity by 42.8% against *P. aeruginosa.* Finally, Rosato *et al.* (2007) reported that the minimum effective dose of norfloxacin was reduced by *Pelargonium graveolens* essential oil against *Bacillus subtilis, Bacillus cereus, Staphylococcus aureus and Escherichia coli.*

In conclusion, different antibiotics used here targeted a variety of sites in bacteria cells and the plant extracts used have different components, which also work on different bacterial target sites. The combination of antibiotics with plant extracts against both Gram-negative and Gram-positive bacteria, including MRSA, may be used to increase their efficacy against a range of infections and also defeat antibiotic resistance. Otherwise, essential oils or some plant extracts should be used with care when combined with antibiotics.

Chapter Six: Comparison of Antimicrobial Activity of Herbal Extracts Alone and in Combination with UV Therapy

6.1 Introduction

6.1.1 UV therapy (phototherapy) for use in combination

As has frequently been mentioned in this Thesis, the development of antibiotic-resistant pathogens is becoming a serious problem, which leads to increasing costs of health care, morbidity, and mortality (Mikulášová *et al*., 2016); this demonstrates the need for novel, cost-effective therapeutic approaches. The use of UV radiation in combination with antimicrobial agents can be considered a promising alternative approach to currently used antibiotics.

Phototherapy was used in ancient India, Greece, and Egypt when a variety of skin diseases for instance, vitiligo was treated by sunlight (Vangipuram and Feldman 2016). Historically, modern therapy was pioneered by Niels Finsen in 1896, who became the father of this approach when he noticed the destructive effect of sunlight on bacteria (Honigsmann, 2013). In Copenhagen, Finsen developed and used ultraviolet light from a carbon-arc lamp to treat more than 800 patients from lupus vulgaris, a skin illness produced by *Mycobacterium tuberculosis* (Honigsmann, 2013; Nee, 1997; Vangipuram and Feldman 2016), and 80% of them were cured (Honigsmann, 2013). Subsequently, in 1903, he was awarded the Nobel Prize in Physiology and Medicine (Honigsmann, 2013; Nee, 1997;

Vangipuram and Feldman, 2016). In 1925, a dermatologist (William Goeckermann) introduced and published his first results on a combination of coal tar and broadband UVB to treat psoriasis (Honigsmann, 2013; Schneider *et al*., 2008). Nearly seventy years later, the groups of Parrish and Wolff used the combination of psoralen and UV-A radiation for psoriasis (Schneider *et al*., 2008; Vangipuram and Feldman 2016).

Basically, sunlight consists of a continuous spectrum of electromagnetic radiation classified into three main wavelengths: visible, ultraviolet (UV), and infrared (Matsumura and Ananthaswamy, 2004); UV radiation is then divided into three components depending on wavelength (UV-A (320 – 400 nm), UV-B (280 – 320 nm) and UV-C (200 – 280 nm) (Matsumura and Ananthaswamy, 2004; Tauchman and Pomory, 2011).

UV-C has a germicidal effect against most types of microbes as a result of absorption of photons by DNA at these wavelengths and the most effective wave lengths are approximately 260-265nm (Gayán *et al*., 2012; Gayán *et al*., 2014). Normally, the stratospheric ozone layer absorbs and blocks UV-C from reaching the Earth's surface (Matsumura and Ananthaswamy, 2004; Narayanan *et al*., 2010; Tauchman and Pomory, 2011), with some 90–99% of UV-A radiation and only 1-10% of UV-B radiation from the solar UVR energy reaching the Earth's surface (Narayanan *et al*., 2010) and causing

serious biological effects on the eyes and skin (Matsumura and Ananthaswamy), the prolonged expose to UVB causes skin cancer and erythema (Gallagher and Lee, 2006; Matsumura and Ananthaswamy, 2004), while UV-A is considered harmless, although prolonged exposure to this radiation could cause aging to the skin (Gallagher and Lee, 2006; Narayanan *et al*., 2010). Thai *et al.* (2005) reported that UV-C light can treat the surfaced layers of chronic wounds through eradicating bacteria, for example *Staphylococcus aureus, methicillin-resistant Staphylococcus aureus* and *Pseudomonas aeruginosa*.

UV light affects bacteria by damaging DNA, being mostly absorbed into the nucleotides; a structure of thymine dimer is created sequentially through two adjacent thymines where the bases on one DNA strand are covalently linked (Goosen and Moolenaar, 2008; Zion *et al*., 2006). The most frequent lesions induced were the formation of cyclobutane pyrimidine dimers, and pyrimidine 6–4 pyrimidone photoproducts (Gayán *et al*., 2014; Matsumura and Ananthaswamy, 2004; Pfeifer *et al*., 2005; Tauchman and Pomory, 2011); as well as RNA and proteins which related to cell membranes (Angélica Garrido-Pereira *et al.,* 2013), the thymine–thymine dimer linkage prevents DNA replication and consequently reproduction (Shang *et al*., 2009); and finally causes cell death (Gayán *et al*., 2014). In addition, in

order to remove UV-induced lesions from the DNA, cells are usually able to repair DNA damage by different DNA repair mechanisms (Goosen and Moolenaar, 2008; Matsumura and Ananthaswamy, 2004). Generally, bacteria can repair DNA lesions in the presence of visible light or not through the two molecular mechanisms of photoreactivation and dark repair (Shang *et al*., 2009; Jungfer *et al*., 2007). Photoreactivation is a mechanism when microorganisms use light from 330– 480 nm to activate a definite enzyme, photolyase, to divide cyclobutane pyrimidine dimers and thus repair the damaged DNA (Shang *et al*., 2009); whereas dark repair, (i.e., nucleotide excision repair), occurs when more than twelve proteins are coordinated, such as helicase which removes the damaged strand and DNA-ligase which is responsible for sealing after repair synthesis (Zion *et al*., 2006) to eradicate the damaged DNA segment (Shang *et al*., 2009). Interestingly, dark repair is more complex and slower than photoreactivation (Shang *et al*., 2009), while photolyase, which is found in bacteria, not in humans, is considered responsible for much of the repair of the UV radiation damage (Zion *et al*., 2006).

The aim of the experimental work described in this Chapter was to determine the antibacterial activity of UV light treatment in combination

with different plant extracts at varying time points and compare these with the antimicrobial activity of plant extracts alone.

6.2 Materials and methods

6.2.1 Sample preparation

Five essential oils (Kanuka oil, Peru balsam oil, Eucalyptus oil, Tea Tree oil and Peppermint oil) and the most promising herbal extracts (Grapefruit seed extract and Goldenseal extract (DL)) samples were generally used directly as purchased.

6.2.2 Test organisms

The following test microorganisms were used: *Staphylococcus aureus*, Methicillin-Resistant *S. aureus* MRSA and *Escherichia coli*.

6.2.3 Inoculum preparation and turbidity standard

The inocula of susceptibility tests were adjusted to $1.5x10^8$ CFU/ml which reference to the (0.5 McFarland standard). Two to five isolated colonies from pure culture were taken using a sterile loop and inoculated into a tube containing distilled, sterile water and mixed by using a vortex mixer until the mixture became homogeneous. The turbidity was then measured using a spectrophotometer at 600 nm. These suspensions were used within 30 minutes of preparation (Andrews, 2001).

6.2.4 Treatment of bacterial suspensions with ultra-violet radiation

A suspension of bacteria was placed into an opened Petri dish and exposed to the UV-A lamps (365 nm, 8 Watt, White, UVL-18, UVP) and UV-B (302nm, 8 Watt, White, UVM-18, UVP) for various times (30s, 60s, 90s and 120s) in a closed box. The distance between the lamps and the surface of the suspension was 6.0 cm. Immediately after UV exposure, 100 µl of the bacterial suspension were poured onto Muller-Hinton agar plates. A sterile glass spreader was used to distribute the inoculum on the surface of agar. All plates were allowed to dry. Wells, 8.0 mm in diameter, were cut from the culture media using a sterile metal cylinder and then filled with a volume of 50µl and 100 µl of plant extracts. A bacterial suspension before exposure with either 50 µl and 100 µl of samples, and the bacterial suspension after exposure without samples, were used as controls. After a 30 min pre-diffusion time interval, the plates were incubated at 37°C for 24 hours and the inhibition zone around each well was measured in mm, including the well (8.0mm). The result was then recorded.

6.2.5 Statistical Analysis

All observations were presented as Mean ± SD (Standard Deviation). The data were analysed by IBM Corp© 24.0. One way ANOVA was performed to compare if there was a significance difference of the inhibition zone

values measured between the different combination of UVR and plant products at various periods against the test organisms. $P \leq 0.05$ was considered as statistically significant. Tukey-Post-Hoc test confirmed the pairwise comparisons.

6.3 Results

The efficacy of the combined processes of UV-light (UV-A and UV-B) and (50µl and 100 µl) of plant products (Kanuka oil, Eucalyptus oil, Peru balsam oil, Tea Tree oil, Peppermint oil, Grapefruit seed extract and Goldenseal) were evaluated by using agar diffusion assay at four different time points of UVR exposure (30, 60, 90 and120 seconds) as presented in Figures 6.3, 6.4, 6.5, 6.6, 6.7 and 6.8. Generally, no difference was observed between the results of the combination of plant products with UV-A and the controls. All bacteria used in the experiments proved to be resistant to UV-A (no data shown), whereas the combined antibacterial effect of UV-B light and plant products showed a significant increase in the size of the inhibition zones compared with the measure of the inhibition zone of plant products alone (Figures 6.3- 6.8). However, a longer UV-B exposure time led to a greater inhibition zone against two of the test pathogens MRSA and *E.coli* as shown in Figures 6.3, 6.5, 6.6 and 6.8, when compared with a shorter exposure time with the same distance (Figure 6.1). *S. aureus* showed a different trend, with all combinations involving 120 seconds exposure, there was a significant reduction in the inhibition zone when compared with the inhibition zone for 90 seconds of exposure to UV-B (Figs.6.2, 6.4 and 6.7). Plant products had a similar trend with different

volumes (100 µl and 50 µl) in this experiment except for Kanuka oil (50µl), which demonstrated neither a bacteriostatic nor a bactericidal effect against *E.coli* with all exposure times, as shown in Figure 6.8; although there was a visible reduction in the density of growth of *E.coli* at different exposure times.

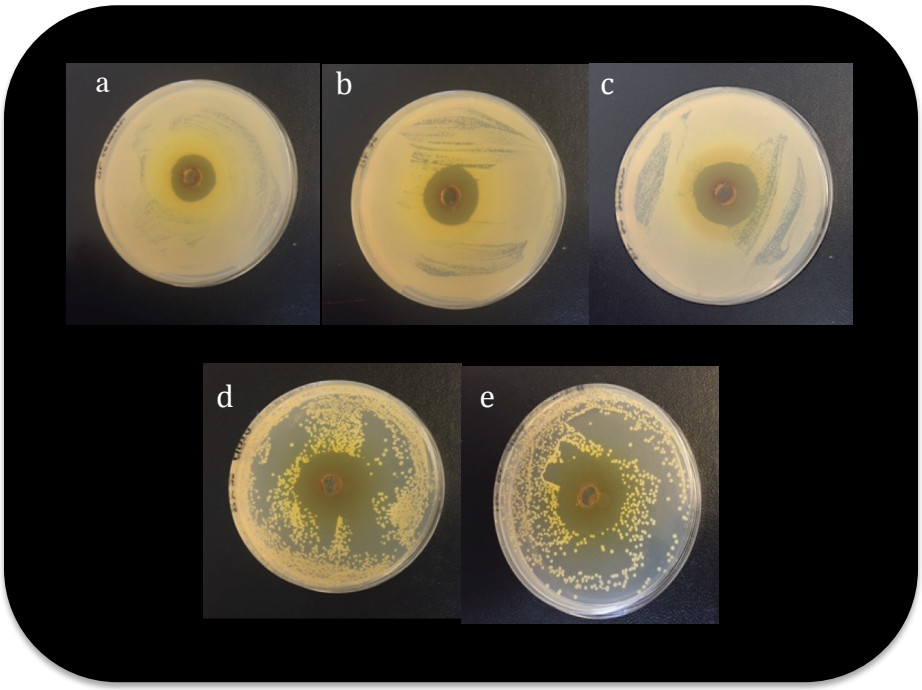

Figure 6.1 Comparison of inhibition zones for Goldenseal (DL.100 µl) after exposure to UV-B at varying time periods: 0, 30, 60, 90 and 120sec. Plates seeded with MRSA: (a) zone of inhibition caused by 0 sec (control), (b) zone of inhibition caused after 30sec of exposure (c) zone of inhibition caused after 60sec of exposure, (d) zone of inhibition caused after 90sec of exposure, (e) zone of inhibition caused after 120sec of exposure.

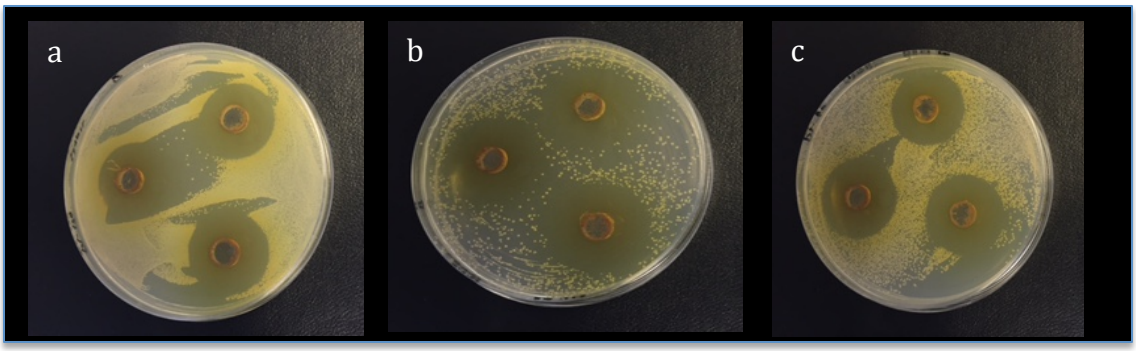

Figure 6.2 Comparison of inhibition zones for Goldenseal (DL.50 µl) after exposure to UV-B at varying time periods: 60, 90 and120sec. Plates seeded with *S. aureus*: (a) zone of inhibition caused by 60 seconds of exposure, (b) zone of inhibition caused by 90sec of exposure (c) zone of inhibition caused by120sec of exposure.

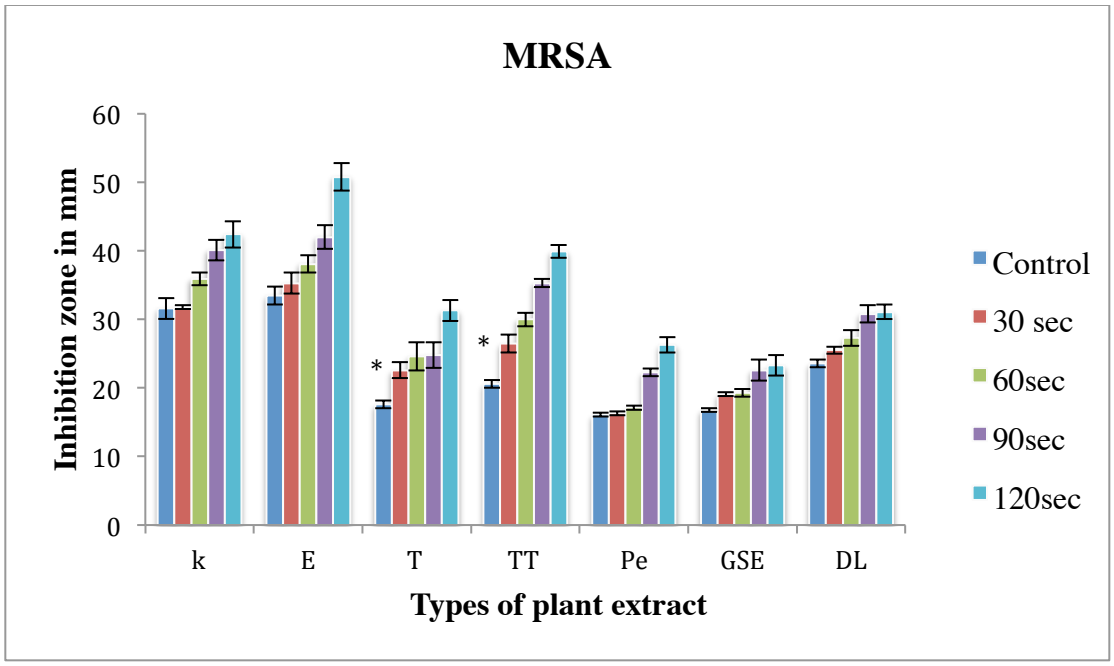

Figure 6.3 Antibacterial properties of UV-B light treatment in combination with different plant extracts at varying durations of time: control (0), 30, 60, 90 and 120 seconds: Inactivation of MRSA upon exposure to 302 nm light and with 100µl of plant extracts. The distance between the lamps and the surface of the suspension was 6.0cm. The values are means of triplicates ± Standard Deviation (including the well (8.0mm)).

*Statistically significant difference between input groups ($p \leq 0.05$).

Kanuka oil= (K)

Peru balsam oil= (Pe)

Eucalyptus oil= (E)

Tea Tree oil= (TT)

Peppermint oil= (T)

Grapefruit seed extract= (GSE)

Goldenseal extract= (DL)

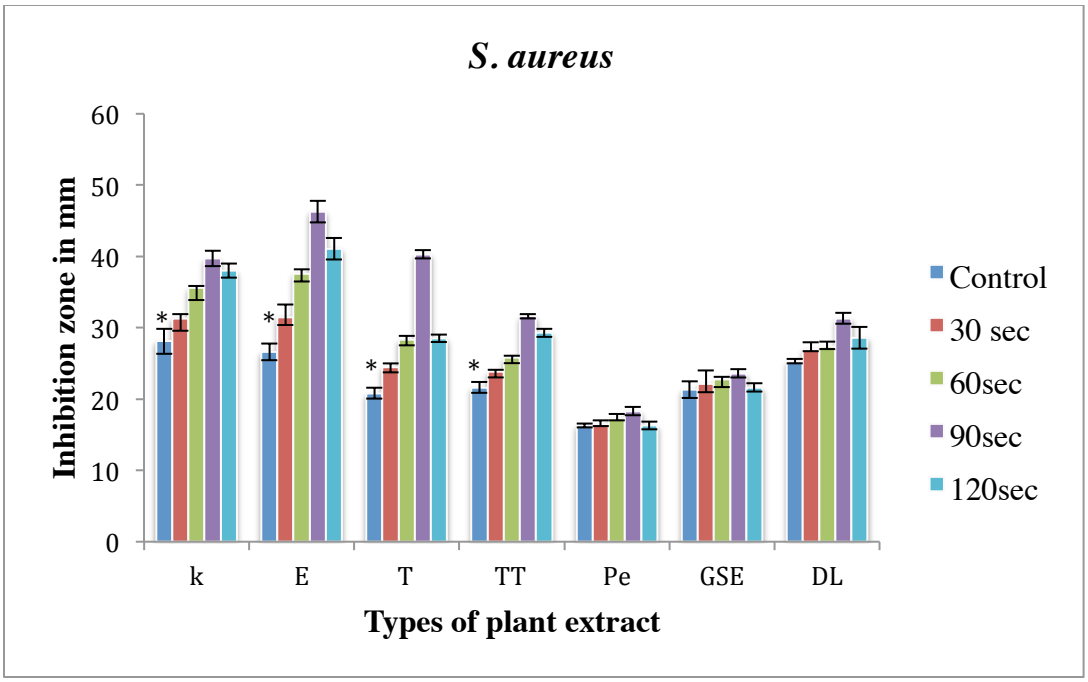

Figure 6.4 Antibacterial properties of UV-B light treatment in combination with different plant extracts at varying durations of time: control (0), 30, 60, 90 and 120 seconds: Inactivation of *S. aureus* upon exposure to 302 nm light and with 100µl of plant extracts. The distance between the lamps and the surface of the suspension was 6.0cm. The values are means of triplicates ± Standard Deviation (including the well (8.0mm)).

*Statistically significant difference between input groups ($p \leq 0.05$).

Kanuka oil= (K)

Peru balsam oil= (Pe)

Eucalyptus oil= (E)

Tea Tree oil= (TT)

Peppermint oil= (T)

Grapefruit seed extract= (GSE)

Goldenseal extract= (DL)

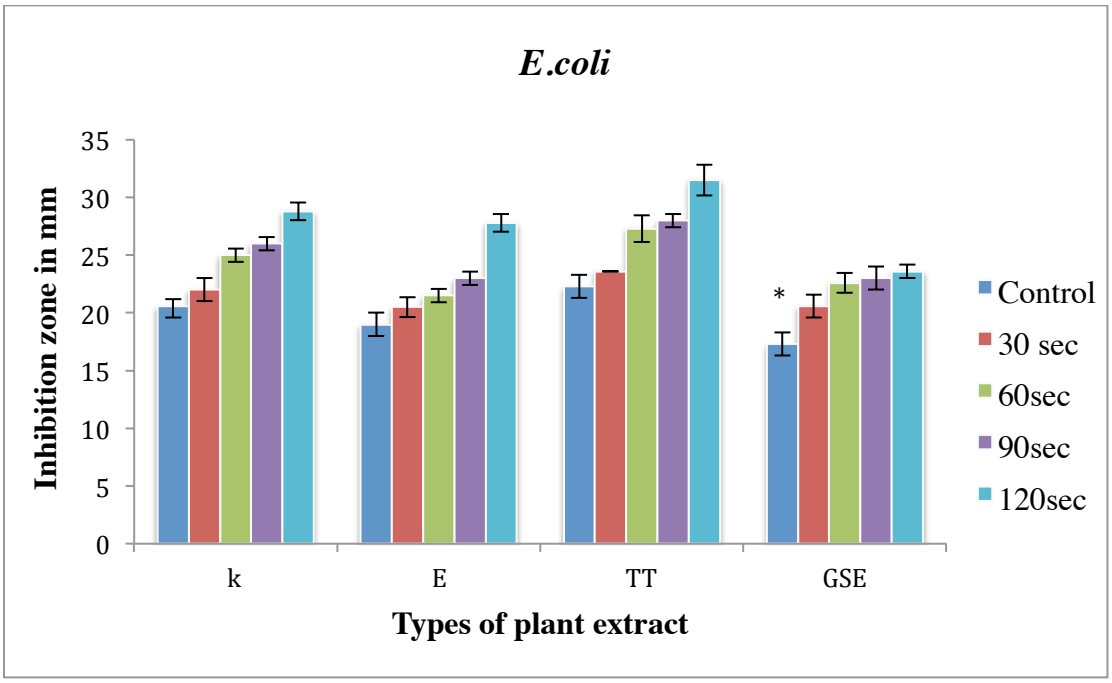

Figure 6.5 Antibacterial properties of UV-B light treatment in combination with different plant extracts at varying durations of time: control (0), 30, 60, 90 and 120 seconds: Inactivation of *E. coli* upon exposure to 302 nm light and with 100µl of plant extracts. The distance between the lamps and the surface of the suspension was 6.0cm. The values are means of triplicates ± Standard Deviation (including the well (8.0mm)).

*Statistically significant difference between input groups ($p \leq 0.05$).

Kanuka oil= (K)

Eucalyptus oil= (E)

Tea Tree oil= (TT)

Grapefruit seed extract= (GSE)

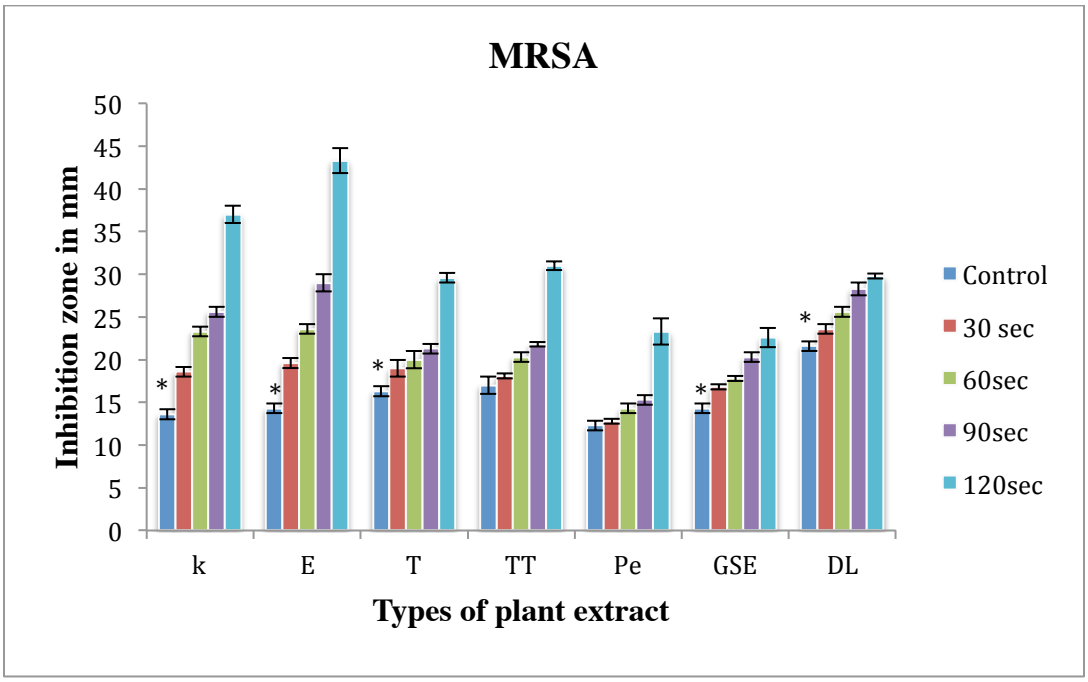

Figure 6.6 Antibacterial properties of UV-B light treatment in combination with different plant extracts at varying durations of time: control (0), 30, 60, 90 and 120 seconds: Inactivation of MRSA upon exposure to 302 nm light and with 50µl of plant extracts. The distance between the lamps and the surface of the suspension was 6.0cm. The values are means of triplicates ± Standard Deviation (including the well (8.0mm)).

*Statistically significant difference between input groups ($p \leq 0.05$).

Peru balsam oil= (Pe)

Eucalyptus oil= (E)

Tea Tree oil= (TT)

Peppermint oil= (T)

Grapefruit seed extract= (GSE)

Goldenseal extract= (DL)

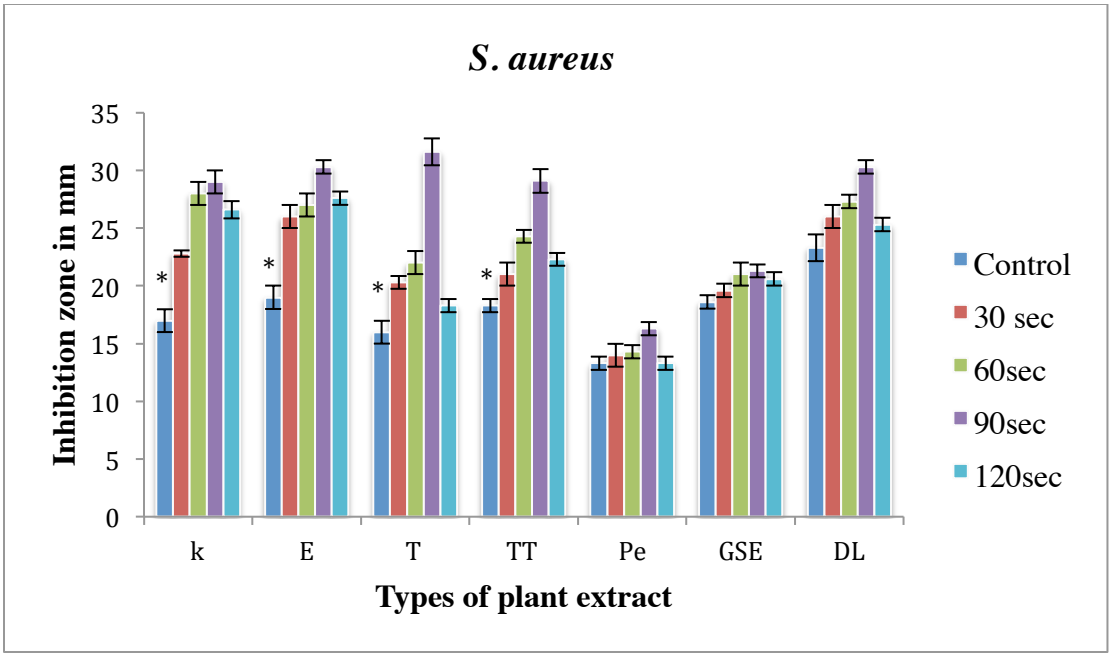

Figure 6.7 Antibacterial properties of UV-B light treatment in combination with different plant extracts at varying durations of time: control (0), 30, 60, 90 and 120 seconds: Inactivation of *S. aureus* upon exposure to 302 nm light and with 50µl of plant extracts. The distance between the lamps and the surface of the suspension was 6.0cm. The values are means of triplicates ± Standard Deviation (including the well (8.0mm)).

*Statistically significant difference between input groups ($p \leq 0.05$).

Kanuka oil= (K)

Peru balsam oil= (Pe)

Eucalyptus oil= (E)

Tea Tree oil= (TT)

Peppermint oil= (T)

Grapefruit seed extract= (GSE)

Goldenseal extract= (DL)

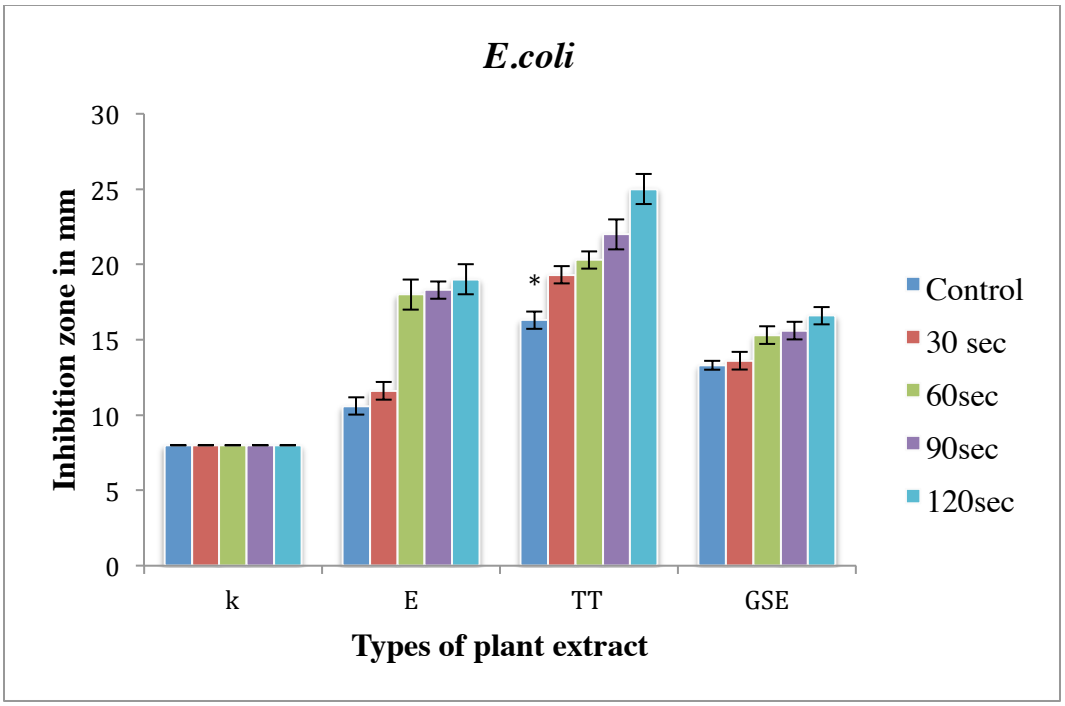

Figure 6.8 Antibacterial properties of UV-B light treatment in combination with different plant extracts at varying durations of time: control (0), 30, 60, 90 and 120 seconds: Inactivation of *E. coli* upon exposure to 302 nm light and with 50µl of plant extracts. The distance between the lamps and the surface of the suspension was 6.0cm. The values are means of triplicates ± Standard Deviation (including the well (8.0mm)).

*Statistically significant difference between input groups ($p \leq 0.05$).

Kanuka oil= (K)

Eucalyptus oil= (E)

Tea Tree oil= (TT)

Grapefruit seed extract= (GSE)

6.4 Discussion

The antimicrobial properties of plant extracts alone and in combination with UVR were studied to compare their abilities to kill bacteria. The results showed that the combination of UV-B and plant extracts exhibited antimicrobial effects against all of the bacteria studied. Although the exposure times were short, UVB caused a bactericidal effect on bacteria even before treated with plant extracts as did not occur with UVA. Dotterud *et al*. (2008) showed that the use of UV-B treatment in patients with atopic dermatitis led to a decrease in the *Staphylococcus aureus* count. In the main, UVR and plant extracts have different modes of actions to inhibit bacteria which is clearly shown by the increase of inhibition zone after combination, and this double attack may explain the increase in the inhibition zone compared with the inhibition zone of plant extracts alone.

Here, the antibacterial properties of UV-A light and plant extracts against MRSA, *S. aureus* and *E.coli* were not different between the combinations and the control samples. This result agrees with those found by Kashiwabuchi *et al.* (2012) who reported that there was no bactericidal effect of the combination of riboflavin 0.1% and ultraviolet light A at 365 nm against oxacillin susceptible *S. aureus*.

As expected, an increase in the period of UV-B exposure produced greater inhibition as shown by the diameter of the inhibition zone. This result was supported by the results of Enwemeka *et al.* (2008) who stated that more bacteria were destroyed with longer radiation periods. When compared with controls (plant extracts alone) there was a significant increase in the inhibition zone following UV-B exposure for all organisms. An exception to this trend was presented by *S. aureus*, which showed a decrease in the size of inhibition zone after 120 seconds compared with 90 seconds of UV-B exposure. Angélica Garrido-Pereira *et al.* (2013) demonstrated that UV radiation exposure can influence microbes through a variety of factors, such as bactericidal wavelength, the period of exposure and the microorganism type.

Generally, Gram-positive bacteria were more susceptible than Gram-negative bacteria when treated with plant extracts as indicated before, also the low amount of Kanuka oil that was applied in this experiment may explain the failure of Kanuka oil (50 µl) to cause neither a bacteriostatic nor a bactericidal effect against *E.coli* through all different exposure times.

In conclusion, **t**he results indicated that application of short periods of exposure to UV weakens bacteria, thereby allowing the plant extracts to

exhibit a greater inhibitory effect. The results suggest that UV should be used in combination with plant extracts to enhance their antibacterial effect.

Chapter Seven: The Liberation of Endotoxin From *E. coli* after being Treated With Herbal Extracts

7.1 Introduction

7.1.1 Endotoxin

Endotoxins are biological toxins produced by Gram-negative bacteria (Abdulraheem *et al.,* 2012). Endotoxin, also called bacterial lipopolysaccharide (LPS), surrounds the cell and forms a major part of the outer membrane of most Gram-negative bacteria (Abdulraheem *et al.,* 2012; Hurley, 2013; Unger *et al.,* 2014; Miao *et al.,* 2013). Each *E. coli* cell contains about 2,000,000 LPS molecules (Magalhães *et al.,* 2007). The term endotoxin was first suggested by Richard Pfeiffer, who in the 1890's differentiated between exotoxin, which is released outside the cell, and endotoxin, which is a cellular component (Hurley, 2013). Endotoxins are very stable and highly resistant to high temperatures or pH values (Magalhães *et al.,* 2007; Unger *et al.,* 2014) and are released when the bacteria cells are destroyed by antibiotics, by host immune cells, or by killing bacteria during the addition of heated solutions or solvents (Unger *et al.,* 2014). In addition, endotoxin can be liberated during bacterial growth and reproduction (Shi *et al.,* 2011; Lodowska *et al.,* 2013). The fact that bacteria can grow in media lacking in nutrients such as saline, buffers and water, means that bacterial endotoxin is ubiquitous (Magalhães *et al.,* 2007).

7.1.2 Biological effects of endotoxin in the host

In the human body, endotoxins do not act against cells or organs directly (Magalhães *et al.,* 2007) but stimulate and activate different immune system receptors (Magalhães *et al.,* 2007; Miao *et al.,* 2013) for example, dendritic, epithelial, platelets, endothelial and leukocytes cells, and also cells of the monocyte-macrophage lineage (Unger *et al.,* 2014). When a small amount of endotoxin (1ng) enters the bloodstream it can cause shivering and fever (Unger *et al.,* 2014). A high dose of liberated LPS (endotoxins), however, produces an inflammatory reaction (Abdulraheem *et al.,* 2012; Morris and Li, 2012), irreversible septic shock, tissue damage, adult respiratory distress syndrome and death (Unger *et al.,* 2014). Abdulraheem *et al.* (2012) noted that the general clinical signs resulting from endotoxin exposure include fever, diarrhea, vomiting, intravascular coagulation, hypotension, septic shock, and death. As a result of these reactions, Magalhães *et al.* (2007) concluded that it is critical that endotoxins be removed from medicines and biological and pharmaceutical materials; endotoxin can for example, be found in pharmaceuticals as a result of the production process. A positive effect has been shown with very low doses of endotoxin ranging between 0.05–0.5 ng/mL which stimulates the immune system to deliver a stronger response to any resultant challenge (Morris and Li, 2012; Lodowska *et al.,*

2013).

7.1.3 Detection of endotoxin

There are several methods for detecting endotoxin, the first of which is the rabbit pyrogen test developed in the 1920s, which, in rabbits, assesses the capability of endotoxin to lead to a temperature rise (Magalhães *et al.,* 2007) and it is sensitive to 0.5 EU/ml (Unger *et al.,* 2014). Another test is the Limulus amoebocyte lysate test. In the 1960s, Levin and Bang described this test to determine endotoxins by employing extracts from blood cells (amoebocytes) of the horseshoe crab (*Limulus polyphemus*) (Abdulraheem *et al.,* 2012; Akbar John *et al.,* 2010). This test includes three components (Miao *et al.,* 2013):

1. The gel-clot method, which is a 60-minute test, is the simplest and highly sensitive. It was the first test approved by the Food and Drug Administration (FDA) (Sharma *et al.,* 2011) and it can detect 0.03 (EU)/ml (Magalhães *et al.,* 2007; Unger *et al.,* 2014).
2. The turbidimetric LAL technique (kinetic based) is based on protein coagulation that occurs when the turbidity is increased relative to the concentration of endotoxin in the sample; it is able to detect a range of concentrations of endotoxin, from 0.01 EU/mL to 100.0 EU/mL

(Magalhães *et al.,* 2007).

3. The chromogenic LAL technique (colorimetric cell-based assay) which is sensitive to concentration lower than 0.3 ng/ml (Unger *et al.,* 2014); it can detect endotoxin by changing the colour of a special dye over a set period of time (Magalhães *et al.,* 2007).

In vitro, the LAL test is the standard quantitative assay for LPS and is the most commonly used test for the detection of endotoxin today (Magalhães *et al.,* 2007; Unger *et al.,* 2014), as well as fluorescence and electrochemical methods (Miao *et al.,* 2013).

7.1.4 Chemical nature of endotoxin (LPS)

Bacterial lipopolysaccharide (LPS) is composed of three distinct regions O-antigen, core oligosaccharide, and lipid A (Lodowska *et al.,* 2013; Magalhães *et al.,* 2007; Opal and Glück, 2003; Raetz and Whitfield, 2002; Shi *et al.,* 2011).

1- The outer polysaccharide (O-antigen) is composed of a sequence of repeating oligosaccharides and each has 3-8 monosaccharides (Magalhães *et al.,* 2007); these repeating sugar units are taxonomically significant as they give bacteria their serological identity and are frequently strain specific (Thorn, 2001; Magalhães

et al., 2007).

2- The core polysaccharide is located in the middle between the oligosaccharide and the lipid A (Thorn, 2001), while the outer region of the core is linked to the O-antigen and the inner region is attached to lipid A via 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) (Lodowska *et al.,* 2013). The Kdo–lipid A region is responsible for endotoxic properties of LPS structure and the growth of bacteria (Lodowska *et al.,* 2013; Raetz and Whitfield, 2002).

3- Lipid A is the hydrophobic part of lipopolysaccharide (Magalhães *et al.,* 2007). It is a glucosamine-based phospholipid (Raetz and Whitfield, 2002). Consequently, Cross (2014); Hurley (2013); Magalhães *et al* (2007); Shi *et al.* (2011) pointed out that the lipid-A component of the lipopolysaccharide is likely to be responsible for the biological activities of endotoxin.

The aim of the experimental work described in this chapter was to detect the ability of various plant products to induce the release of bacterial endotoxin from *E. coli*.

7.2 Material and methods

In order to identify the liberation of endotoxin from Gram-negative bacteria (*E. coli*) under the influence of the addition of plant products, endotoxin was detected by the simplest form of Limulus Amebocyte Lysate LAL assay which is the Gel-Clot Assay. A commercially available assay, the Gel Clot Endotoxin Assay Kit (ToxinSensor™, GenScript) was used. All materials used for sample preparation and test reagent preparations were endotoxin-free. A aseptic technique was used at all times. LAL-endotoxin reaction is pH dependent; a useable pH range for the Limulus assay was considered to be between pH 6.0-8.0. Endotoxin-free sodium hydroxide solution 0.1N (Sigma Aldrich) or endotoxin-free hydrochloric acid solution 0.1N (Sigma Aldrich) were used to adjust the pH level.

7.2.1 Sample preparation

Five herbal extracts (Grapefruit seed extract, Goldenseal extract (DL), Goldenseal extract (D), Derum and Noni juice) and five essential oil (Kanuka oil, Eucalyptus oil, Peru balsam oil, Tea Tree oil and Peppermint oil) samples were generally used undiluted.

7.2.2 Turbidity standard for inoculum preparation

The inocula was adjusted to $1.5x10^8$ colony forming units (CFU), which equals 0.5 McFarland. Two to five isolated colonies of the same morphological type were taken with a sterile loop and inoculated into a tube containing reagent water endotoxin-free and then mixed by a vortex mixer until they became homogenized and then, using a spectrophotometer at 600 nm. This suspension was used within 30 minutes of preparation (Andrews, 2001).

7.2.3 Reagent preparation

All the following steps were conducted according to the manufacturer's instructions (ToxinSensor™, GenScript).

7.2.3.1 Preparation of Limulus Amebocyte Lysate (LAL) solution

Lyophilized lysate was reconstituted by adding 2 ml of endotoxin-free water and was swirled gently for at least 30 seconds until all the solid contents dissolved thoroughly. The lysate solution was then stored at -20°C or below for up to one week.

7.2.3.2 *E. coli* endotoxin standard

E.coli Endotoxin Standard 0.5 EU/ml was reconstituted by adding 1 ml of LAL reagent water and mixed thoroughly for at least 15 minutes with a

vortexer in order to obtain an endotoxin stock solution. Reformed endotoxin standard was stored at -20°C or below for up to 15 days.

7.2.4 Test procedure

(0.1 ml) of a sample of the plant product containing *E.coli* was added after incubation for 24 hours to release endotoxin to 0.1 ml of *Limulus* amoebocyte lysate solution in disposable endotoxin-free glass test tubes in order to detect the release. The tubes were capped and the contents mixed thoroughly and incubated at 37°C for 60 minutes. After incubation, the presence of a gel clot was considered to be a positive result. Four types of control were set up with LAL:

1. (PC): Positive control using *E. coli* endotoxin standard (0.5 EU/ml) only.
2. (NC): Negative control using LAL reagent water only.
3. (NPC): Negative product control containing plant product test samples.
4. (PPC): Positive product control containing equal amounts of *E. coli* endotoxin and plant product test samples.

7.3 Results

In order to detect the liberation of endotoxin, when LAL solution is added to plant product samples containing endotoxin; a gel will be formed to the endotoxin sensitivity of 0.25 EU/ml or more. A total of 10 different plant products were used (Table 7.1). In nine out of ten cases, no gel was formed, and only Tea Tree oil (Fig. 7.2) (Table 7.1) detected endotoxin and gelled following 60 minutes incubation. None of the negative product controls (NPC) containing plant product test samples formed gel while the formation of gel was recorded in almost all positive product controls containing equal amounts of *E. coli* endotoxin and plant product test samples (PPC) with the exception of Goldenseal and Peru balsam oil (Table 7.1).

Generally, the presence of a hard gel clot was considered a positive test for endotoxin (Fig. 7.1), meaning that the endotoxin concentration in the tube was more than or equal to the sensitivity of 0.25 EU/ml. However, results which showed an increase in viscosity, turbidity and clear liquid were considered a negative test (Fig. 7.1), which showed that the concentration of endotoxin was less than the sensitivity of LAL reagent or that no endotoxin was present.

Table7.1 Summary of the results for the gel clot assay on the plant product samples at an endotoxin concentration of 0.25 EU/ml.

Plant product samples	Gel clot assay	NPC	PPC
Kanuka oil	_	_	+
Grapefruit seed extract	_	_	+
Noni juice	_	_	+
Tea Tree oil	+	_	+
Eucalyptus oil	_	_	+
Derum	_	_	+
Goldenseal (DL)	_	_	_
Goldenseal (D)	_	_	+
Peppermint oil	_	_	+
Peru balsam oil	_	_	_

NPC: Negative product control containing plant product test samples.

PPC: Positive product control containing equal amounts of *E. coli* endotoxin and plant product test samples.

+: Gel formed.

-: No gel formed.

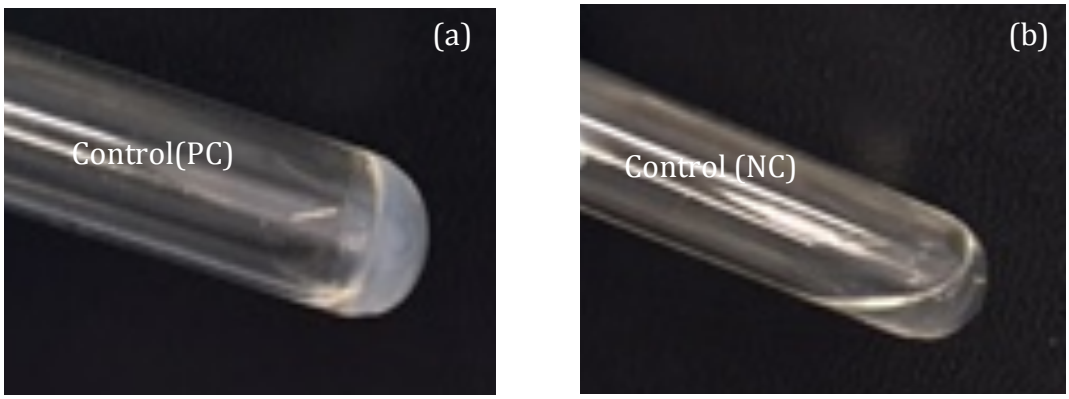

Figure7.1: a: (PC): Positive control using *E. coli* endotoxin standard (0.5 EU/ml); b: (NC) negative control using LAL reagent water.

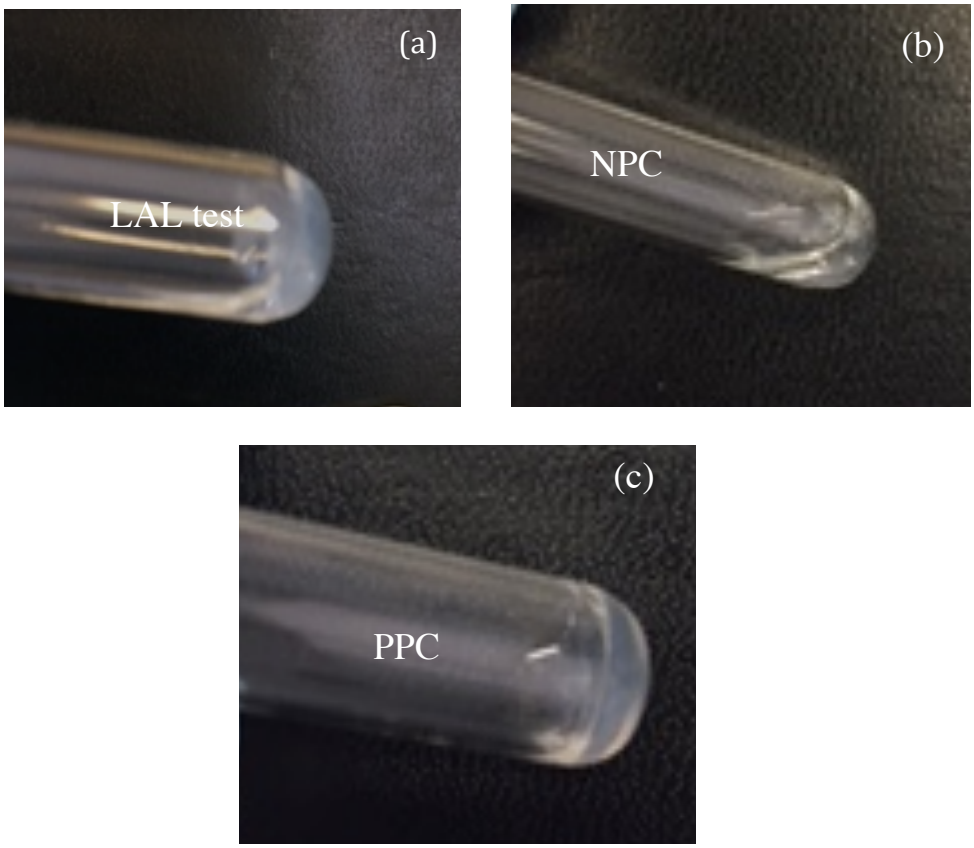

Figure7.2: The results for the gel clot assay (LAL assay) on (a): Tea Tree oil at an endotoxin concentration of 0.25 EU/ml. (b): NPC: negative product control containing Tea Tree oil test samples; (c): PPC: positive product control containing equal amounts of *E. coli* endotoxin and Tea Tree oil test samples.

7.4 Discussion

Due to only slight errors in positive and negative test results, Sharma *et al*. (2011) concluded that in injectable radiopharmaceutical products the gel clot assay is regarded as the most accurate and sensitive method for detecting endotoxin.

The results presented in this thesis show that only one of the 10 samples formed a gel while the others did not; this could be due to the fact that these samples contain endotoxin less than 0.25 EU/ml, or possibly that these products do not liberate endotoxin from *E. coli*. The most surprising result to appear from this experiment is that Peru balsam oil and Goldenseal with pure endotoxin did not form a gel while, and when pure endotoxin was added without Peru balsam oil and Goldenseal, a gel formed. The inhibition of the gel formation may be due to the presence of chemical components of Peru balsam oil and Goldenseal, which act as inhibitory components. This finding agrees with the study by Sharma *et al.* (2011), which showed the inhibition of the gel formation in some samples is due to the presence of the citrate ion.

In conclusion, while it is clear from previous results that plant extracts kill bacteria in general, no measurable endotoxin was released in almost all

results. This finding can obviously be regarded as being beneficial.

Chapter Eight: Final Discussion

8.1 Final Discussion

The world's flora comprise a large number of medical and aromatic plants (Swamy *et al*., 2016), which represent an important medical resource (Rivera *et al.,* 2013). These natural medicines are used all over the world (including parts, extracts etc.) in curing and avoiding specific ailments and diseases (Nwachukwu *et al.,* 2010). Herbal medicine use dates back some 5,000 years (Rivera *et al.,* 2013) and today; some eighty percent of the world's people use plant-derived medicines (Atul Bhattaram *et al.,* 2002), which reach some ninety-five percent in the developing world (Rivera *et al.,* 2013). Some nine thousand wild plants have now been recognized as curative agents (Swamy *et al*., 2016). Clearly, medicinal plants have been shown, over an extended period, to be safe and effective while exhibiting few side effects (Kamboj, 2000). In fact, these plants form the bedrock of modern medicine (Pal and Shukla, 2003), such that many conventional drugs came from plants (Pal and Shukla, 2003). At present, around twenty-five percent of US pharmaceutical prescriptions contain at least one plant-derived component (Verma and Singh, 2008), and in excess of sixty plants are known to exhibit useable antibacterial agents (Verma and Singh, 2008), active against both strains which are drug-sensitive and drug-resistant (Raut and Karuppayil, 2014, Ncube *et al.,* 2008, Sokovic *et al.,* 2010), including

bacteria, fungi or viruses (Moghadam *et al.,* 2010). Herbalists mainly use unpurified plant extracts which contain a range of constituents, since these, it is claimed, are more effective because the components making up the extract work synergistically so that the effect of the whole product is more than the sum of its parts, and any toxicity is markedly reduced when the total herb is used compared to its isolated active ingredients (Pal and Shukla, 2003).

The severity of bacteria-induced infections has increased of late due to a) the emergence of antibiotic resistant strains of bacteria, b) an increase in the population exhibiting overall lowered immunity and finally, c) an increase in the occurrence of infections associated with the development of antibiotic-resistant biofilms (Raut and Karuppayil, 2014). The emergence of multi-resistant bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant Enterococci is particularly worrisome (Rudramurthy *et al*., 2016), and there is an urgent need for alternative approaches for use to defeat human pathogens (Raut and Karuppayil, 2014).

Plant–derived molecules often inhibit Gram-positive and Gram-negative bacterial pathogens (Raut and Karuppayil, 2014) and the use of essential plant oils for the treatment of cases of epidemic multidrug-resistant

infections shows considerable potential (Mulyaningsih *et al*., 2010; Swamy *et al*., 2016).

In this study, attention was paid to herbal therapy and combination therapy and an evaluation was made of the *in vitro* antimicrobial activities of nineteen plant extracts against Gram-negative bacteria and Gram-positive bacteria including methicillin-resistant *S. aureus* (MRSA) and two species of yeast (*Candida rugosa* and *Candida inconspicua*). The antimicrobial activity of each plant extract was evaluated alone and in various combinations. Out of nineteen plant extracts tested, five essential oils and five herbal extracts showed antimicrobial activity against almost all of the microorganisms studied. Kanuka oil, Eucalyptus oil, Tea Tree oil, Goldenseal and Grapefruit seed extract exhibited the greatest antibacterial activity. Kanuka oil showed the most potent activity against most of the microorganisms studied, especially MRSA. The antibacterial properties of plant extracts showed a significant increase in the measure of the inhibition zone following UV-B exposure compared with the measure of the inhibition zone of plant products alone. Generally, an increase in the period of UV-B exposure produced greater inhibition as shown by the diameter of the inhibition zone. An exception to this trend was shown by *S. aureus*. Plant extracts also improved the efficacy of almost all antibiotics and a

mixture of plant extracts and antibiotics proved more inhibitory effects than antibiotics alone. In one out of ten cases, only Tea Tree oil released endotoxin from *E.coli.* It is therefore suggested that these oils could be used as a novel way to combat bacterial resistance to antibiotics.

Essential oils comprise complex secondary metabolites in the form of volatile molecules (Faleiro, 2011), although such essential oils are not "true oils" because they lack lipids (Yap *et al.,* 2014), The chemical constituents of plant essential oils obviously differ between species and such differences directly impact on their antimicrobial properties against pathogenic microbes (Swamy *et al*., 2016). Most contain terpenes and aromatic compounds (Yap *et al.,* 2014). The effectiveness of essential oils differs from one type to another as well as against different target bacteria depending on whether they are Gram-positive and Gram-negative (Swamy *et al*., 2016). Swamy *et al.* (2016) observed that the highest antimicrobial activity of essential oils is seen against Gram-positive rather than Gram-negative bacteria. For example, Tongnuanchan and Benjakul (2014) found that oregano essential oil inhibits Gram-positive bacteria (*S. aureus*) more than gram-negatives (*E. coli* and *Pseudomonas aeruginosa*). Sandalwood and Vetiver oils exhibit high antimicrobial activity against Gram-positive bacteria but fail to inhibit Gram-negatives (Swamy *et al.,* 2016); both

findings agree with the results reported in this Thesis.

The primary target of EOs against bacteria is the cytoplasmic membrane, disruption and permeabilization of which leads to the loss of important cellular functions such as ion homeostasis and electron transport chain (Raut and Karuppayil, 2014); the hydrophobicity of EOs allows them to partition lipids of the cell membrane (Burt, 2004; Rodrigues *et al.,* 2009) and mitochondria, making them permeable and thereby producing cell – leakage and death (Burt, 2004). Swamy *et al.* (2016) demonstrated that tea tree oil inhibits the growth of *S. aureus* and *E. coli* by altering cell permeability, leading to an increase of intracellular K^+ ion-leakage.

In yeasts and fungi, essential oils coagulate the cellular components due to damage to cell membranes (Swamy *et al.,* 2016), while alterations in membrane fluidity lead to leakage of the contents of the cytoplasm and result in a marked loss of viability. Such membrane permeability and changes in respiratory chain activity in *C. albicans* cells is fatally inhibited following treatment with tea tree oil (Raut and Karuppayil, 2014).

The combination of different plant extracts or plant extracts with conventional antibiotics also shows promise (Chanda and Rakholiya, 2011). The possible benefits of using combination therapy as compared to

monotherapy are a reduction in the emergence of resistant microbes, enhanced efficiency, increases in the stability and or bioavailability of the free agents, a reduction in any undesirable effects, a reduction in the time needed for long-term antimicrobial therapy, the therapeutic effect being produced by using relatively small doses and an ability to treat mixed infections (Aiyegoro and Okoh, 2009; Chanda and Rakholiya, 2011; Darwish *et al*., 2002; Tngdn, 2014). Several *in vitro* studies have shown synergistic effects resulting from a combination of antibiotics with various unpurified plant extracts**,** against *Staphylococcus aureus* strains as well as Gram-negative (Adwan *et al*., 2010). Ethanolic extracts of *Isatis tinctoria*, *Rheum palmatum* and *Scutellaria baicalensis* can increase the antimicrobial activity of ciprofloxacin, ceftriaxone, penicillin G and gentamicin (Zai-Chang *et al.,* 2005), while the interaction between penicillin G and tea extracts proves to be essentially additive against *S. aureus* (Esimone *et al.,* 2006)**.** It has also been demonstrated that the essential oil of *Pelargonium graveolens* reduces the minimum effective dose of norfloxacin against *Staphylococcus aureus, Bacillus subtilis, Bacillus cereus* and *Escherichia coli* (Rosato *et al.,* 2007), and these findings agree with the previous results reported in this Thesis.

While some combinations of plant extracts studied for the work described

in this Thesis produced a synergistic effect against microorganisms; the combinations between Goldenseal extract (DL) and Eucalyptus oil produced a synergistic effect against *Candida rugosa* and *Candida inconspicua;* this synergistic effect was also seen when combined Goldenseal extract (DL) and Peppermint oil was tested against *Candida inconspicua.* Antagonistic effects of different Eos or Eos with antibiotics can also be seen. For example a positive synergestic effect of such a combination of clove oil and rosemary oil was seen against *Candida albicans,* but an antagonistic effect was observed against *Aspergillus* (Faleiro, 2011).

The antibacterial properties of plant extracts were shown here to significantly increase the size of the inhibition zone following UV-B exposure compared with the inhibition zone of plant products alone. The occurrence of MRSA, has led to studies on the antibacterial activity of a broad range of novel biological and chemical compounds, i.e. so-called adjunctive or alternative therapies (Kashiwabuchi *et al*., 2012). UV light, for example, effectively kills bacteria, viruses, and parasites, while forming limited disinfection side products (Jungfer *et al*., 2007). Ultraviolet (UV) radiation phototherapy has long been used to treat common skin diseases (Krutmann *et al.,* 2005; Matsumura and Ananthaswamy, 2004; Vangipuram

and Feldman, 2016). UV-radiation causes biosynthesis failures in bacteria, which lead to cell death (Kashiwabuchi *et al*., 2012). UV radiation can also damage DNA (Angélica Garrido-Pereira *et al.,* 2013) following its absorption by the double bond in the pyrimidine bases of DNA (i.e. thymidine and cytosine); UV adsorption opens such bonds, allowing the UV-modified base to react with nearby bases (Enwemeka *et al*., 2008), resulting in direct killing or defects in growth rates (Angélica Garrido-Pereira *et al.,* 2013). In addition, it can damage cell-membrane proteins, which also significantly impact on growth and reproduction (Angélica Garrido-Pereira *et al.,* 2013). In some cases however, cells can repair the resulting photo-damage of DNA (Enwemeka *et al*., 2008).

In the studies reported here, no significant difference was found between autoclaved and non-autoclaved activities for almost all herbal extracts. This is particularly important with regard to the possible use of herbal extracts to treat wounds, as it is important that they should be sterilized before being used for this purpose; sterilization of herb extracts by autoclaving provides an inexpensive and readily available way to achieve sterilization without inducing a marked change in its antibacterial effects. However, an autoclaved, sterilized, commercially available Kanuka oil and Eucalyptus oil should be clinically avoided for the treatment of wounds which are

infected with bacteria, particularly MRSA. Azu *et al.* (2006) reported that many of the components of medicinal plants and extracts are heat-labile, for example the antibacterial components of the spice plant are destroyed by heat, while all the antibacterial properties of spices are lost when they are heated at 20 minutes at 100°C. In the case of the antimicrobial substance in onion extracts, thermal-destruction of phenolics explains why the antimicrobial activity is destroyed by heating (Azu *et al.*, 2006). The differing results obtained for each individual plant extracts suggest that heat impairs the antibacterial activity of herbal extracts in a variety of ways; that could be due to differences in their components; this fact should be considered before using heat as a sterilant.

In conclusion, the results of this Thesis show that plant extracts offer considerable promise in treatment and management of infections produced by antibiotic resistant bacteria and other nosocomial bacteria and microbes in general. While efforts with regards to defeating antibiotic resistant bacteria are largely focused on complex molecular approaches and to the search for new antibiotics, it is hoped that the effectiveness of plant extracts in killing pathogens will not be overlooked.

It is important note that the results often varied when new bottles of oil

were used. This fact is concerning in relation to the potential application of the oils studied here in medicine and clearly some form of standardization is required before the oils can be medically used. The lack of standardization may explain the differences reported for the antimicrobial effect of essential oils in the literature.

Nevertheless, the essential oils used here are cheap and are globally available. As has been shown in this thesis, important pathogens are vulnerable to a wide range of plant extracts. This study suggests that EOs and other plant extracts therefore provide a promising way forward as an alternative for the treatment of localized infections.

8.2 Suggestions for future work

Two obvious possibilities for future research arise from the data presented here, namely:

1) There is a need to further investigate the effectiveness as antibacterial agents, of herbs and their oils from plants collected from all over the world, including novel species obtained from otherwise unexplored regions.

2) There is a clear need for clinical trials to be conducted on the oils shown here to have the most marked antimicrobial activity.

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Appendices

Appendix A

Manufacturer information of UV lamps

EL Series Ultraviolet Hand Lamps
User's Guide

Introduction

The EL Series of Ultraviolet Lamps offer a uniform and intense source of ultraviolet light (radiation). The lamps emit one of three UV wavelengths or white light in four watt, six watt and eight watt configurations for exposure/illumination of materials. All EL Series Lamp models can be used with the universal J138 Lamp Stand. The four watt models can be used with C-10E Cabinet for viewing materials in a darkroom environment. Eight watt lamps can be used with the C-65 Cabinet.

Important Safety Information

Caution: Shortwave and midrange UV radiation will cause damage to unprotected eyes and skin. Before operating any unit, be sure all personnel in the area are properly protected. UV Blocking Eyewear should be worn as well. UVP has a complete line of UV Blocking Eyewear: Spectacles, Goggles and Faceshield designed for this purpose.

Operation

Plug power cord into a properly grounded electrical outlet. The proper voltage of the lamp is found on the product information label.

A rocker switch is conveniently located on top of the unit to turn the lamp on or off. For models with multiple wavelengths, the rocker switch accommodates for the selection of wavelength or to shut the unit off.

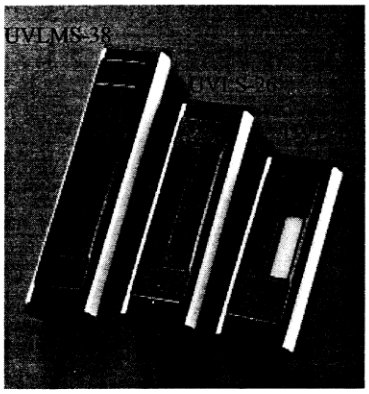
Each lamp comes with two UV tubes or one UV and one white light tubes; the UVLSM-38 is equpped with three tubes. Models with two tubes of the same wavelength operate with both tubes on at same time. Other models operate with only a single tube at one time.

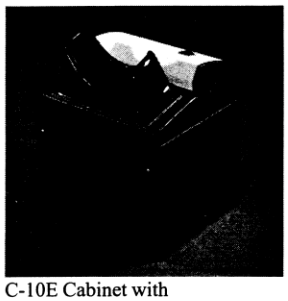
Specifications

Physical dimensions for four, six and eight watt lamps:

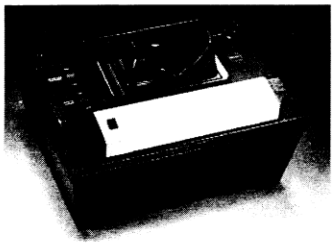
Four watt lamps:	9.8"L x 2.5"H x 3.8"W (249 x 64 x 97 mm)
Six watt lamps:	11.9"L x 2.5"H x 3.8"W (302 x 64 x 97 mm)
Eight watt lamps:	14.9"L x 2.5"H x 3.8"W (376 x 64 x 97 mm)

UVP
ULTRA-VIOLET PRODUCTS

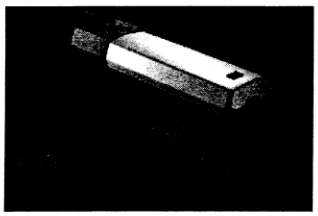




C-10E Cabinet with
four watt lamp



C-65 Cabinet with
eight watt lamp



Universal stand for all
EL Series Lamps

Four Watt Lamp Specifications

Model	Wavelength	Part Number	Nominal Volts/Hz/Amp
UVS-14	254nm/White Light	95-0266-01 95-0266-02 95-0266-03	115/60/0.16 230/50/0.16 100/50-60/0.16
UVL-14	365nm/White Light	95-0264-01 95-0264-02 95-0264-03	115/60/0.16 230/50/0.16 100/50-60/0.16
UVS-24	254nm	95-0269-01 95-0269-02 95-0269-03	115/60/0.32 230/50/0.32 100/50-60/0.32
UVL-24	365nm	95-0267-01 95-0267-02 95-0267-03	115/60/0.32 230/50/0.32 100/50-60/0.32
UVLS-24	365nm/254nm	95-0271-01 95-0271-02 95-0271-03	115/60/0.16 230/50/0.16 100/50-60/0.16

Six Watt Lamp Specifications

Model	Wavelength	Part Number	Nominal Volts/Hz/Amp
UVS-16	254nm/White Light	95-0274-01 95-0274-02 95-0274-03	115/60/0.16 230/50/0.16 100/50-60/0.16
UVL-16	365nm/White Light	95-0272-01 95-0272-02 95-0272-03	115/60/0.16 230/50/0.16 100/50-60/0.16
UVM-16	302nm/White Light	95-0273-01 95-0273-02 95-0273-03	115/60/0.16 230/50/0.16 100/50-60/0.16
UVS-26	254nm	95-0277-01 95-0277-02 95-0277-03	115/60/0.32 230/50/0.32 100/50-60/0.32
UVL-26	365nm	95-0275-01 95-0275-02 95-0275-03	115/60/0.32 230/50/0.32 100/50-60/0.32
UVM-26	302nm	95-0276-01 95-0276-02 95-0276-03	115/60/0.32 230/50/0.32 100/50-60/0.32
UVLS-26	365nm/254nm	95-0279-01 95-0279-02 95-0279-03	115/60/0.16 230/50/0.16 100/50-60/0.16
UVLM-26	365nm/302nm	95-0278-01 95-0278-02 95-0278-03	115/60/0.16 230/50/0.16 100/50-60/0.16

Eight Watt Lamp Specifications

Model	Wavelength	Part Number	Nominal Volts/Hz/Amp
UVS-18	254nm/White Light	95-0200-01 95-0200-02 95-0200-03	115/60/0.16 230/50/0.16 100/50-60/0.16
UVL-18	365nm/White Light	95-0198-01 95-0198-02 95-0198-03	115/60/0.16 230/50/0.16 100/50-60/0.16
UVM-18	302nm/White Light	95-0199-01 95-0199-02 95-0199-03	115/60/0.16 230/50/0.16 100/50-60/0.16
UVS-28	254nm	95-0249-01 95-0249-02 95-0249-03	115/60/0.32 230/50/0.32 100/50-60/0.32
UVL-28	365nm	95-0248-01 95-0248-02 95-0248-03	115/60/0.32 230/50/0.32 100/50-60/0.32
UVM-28	302nm	95-0250-01 95-0250-02 95-0250-03	115/60/0.32 230/50/0.32 100/50-60/0.32
UVLS-28	365nm/254nm	95-0201-01 95-0201-02 95-0201-03	115/60/0.16 230/50/0.16 100/50-60/0.16
UVLM-28	365nm/302nm	95-0251-01 95-0251-02 95-0251-03	115/60/0.16 230/50/0.16 100/50-60/0.16
UVLMS-38	365/302/254nm	95-0252-01 95-0252-02 95-0252-03	115/60/0.16 230/50/0.16 100/50-60/0.16

Replacement Tubes and Switches

Replacement Part	Part Number
Switch, On/Off	53-0134-01
Switch, On/Off/On	53-0135-01
Tube, 4 watt, 365nm	34-0005-01
Tube, 4 watt, 254nm	34-0066-01
Tube, 4 watt, white light	34-0003-01
Tube, 6 watt, 365nm	34-0034-01
Tube, 6 watt, 302nm	34-0044-01
Tube, 6 watt, 254nm	34-0015-01
Tube, 6 watt, white light	34-0063-01
Tube, 8 watt, 365nm	34-0006-01
Tube, 8 watt, 302nm	34-0042-01
Tube, 8 watt, 254nm	34-0007-01
Tube, 8 watt, white light	34-0056-01

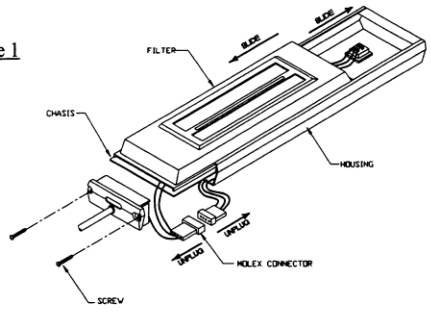
Accessories

Accessories	Part Number
J138 Lamp Stand	18-0063-01
C-65 Cabinet	95-0257-01
C-10E Cabinet	95-0072-08
Spectacles	98-0002-01
Goggles	98-0002-02
Faceshield	98-0002-04

Changing the Replacement Tubes

Always disconnect the lamp from the electrical power source prior to replacing tubes. Remove the two Phillips Head Screws located in the End Cap. Grasp the extruded housing and slide the chassis and filter out together away from the switch. Disconnect the interior Molex Connector (see Figure 1) and slide chassis and filter out of the housing completely. Once out of the extrusion, the chassis and filter will come apart. Carefully grasp the tube ends and twist one quarter turn until it works free (see Figure 2). Insert the new tube, giving it a twist to lock in place. Reassemble the lamp. Be careful not to pinch wires during assembly.

Figure 1



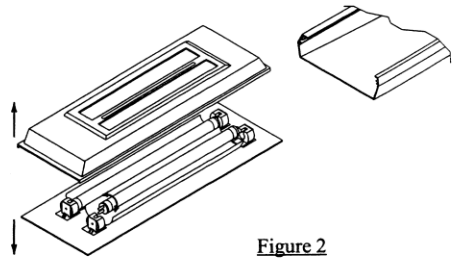


Figure 2

Cleaning the EL Series Lamps

The painted surfaces and filter areas of the lamp should cleaned with a damp sponge or cloth towel and mild soap. Never use abrasive cleaners, solvent based cleaners or scouring pads.

ALWAYS DISCONECT THE LAMP FROM THE ELECTRICAL POWER PRIOR TO CLEANING.

Maintenance/Repair/Technical Assistance

UVP offers technical support for all of its products. If you have any questions about the product's use, operation or repair, call or fax UVP Customer Service at the following offices:

In the US: Tel: (909)946-3197 or toll free (800)452-6788; Fax (909)946-3597; E-Mail uvp@uvp.com
Europe/UK: Tel: +44(0)1223-420022; Fax: +44(0)1223-420561; E-Mail: uvp@dial.pipex.com

A **Returned Goods Authorization (RGA)** number must be obtained from UVP Customer Service before returning any products.

Warranty

UVP, Inc. warrants its EL Series Lamps to be free of defects in materials and workmanship for a period of one (1) year from the date of purchase. Tubes and filters are warranted for a period of 90 days. If equipment failure or malfunction occurs durng the warranty period, UVP shall examine the inoperative equipment and have the option of repairing or replacing any part(s) which, in the judgement of UVP, were originally defective or became so under conditions of normal usage and service.

No warranty shall apply to this instrument, or part thereof, that has been subject to accident negligence, alteration, abuse or misuse by the end user. Moreover, UVP makes no warranties whatsoever with respect to parts not supplied by UVP or that have been installed, used and/or serviced other than in strict compliance with the instruments appearing in this manual.

In no event shall UVP be responsible to the end user for any incidental or consequential damges, whether foreseeable or not, including but not limited to property damage, inability to use equipment, lost business, lost profits, or inconvenience arising out of or connected with the use of instruments produced by UVP. Nor is UVP liable or responsible for any personal injuries occurring as a result of the use, installation and/or servicing of equipment.

This warranty does not supersede any statutory rights that may be available in certain countries.

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81-0117-01 Rev. A

Appendix B

Manufacturer information of Gel Clot Endotoxin Assay Kit

GenScript Make Research Easy

ToxinSensor™ Gel Clot Endotoxin Assay Kit **Cat. No. L00351**

Technical Manual No. 0356 **Version 02072012**

I DESCRIPTION

GenScript **ToxinSensor™ Gel Clot Endotoxin Assay Kit** is designed as a simple and sensitive *in vitro* end-product endotoxin test for human and animal parenteral drugs, biological products, and medical devices. The Limulus Amebocyte Lysate (LAL) test is a qualitative test for Gram-negative bacterial endotoxin. Limulus Amebocyte Lysate supplied in the kit needs to be reconstituted with LAL Reagent Water and then mixed in equal parts with the solution being tested. After incubation, and in the presence of endotoxin, gelation occurs; in the absence of endotoxin, gelation does not occur. The kit contains all the necessary reagents and endotoxin-free materials. The sensitivity of LAL in this kit is 0.25 EU/ml. The protocol described herein conforms to those described in the FDA guideline. Similar performance requirements for gel clot assays have been published and are updated regularly in the United States Pharmacopeia.

II KIT CONTENTS

Components	L00351
Size	40 Assays
Limulus Amebocyte Lysate (LAL), 2 ml/vial	2 vial
LAL Reagent Water, 10 ml/bottle	4 bottles
E. coli Endotoxin Standard, 0.5 EU/vial	2 vials
Endotoxin-free vial	5 × 16
Endotoxin-free Tips, 200 µl	1 box (96 tips)
Endotoxin-free Tips, 1000 µl	2 bags (12 tips)
Incubation Rack	1

III MATERIALS AND EQUIPMENT NOT PROVIDED

860 Centennial Ave., Piscataway, NJ 08854, USA
Tel: 1-732-885-9188 Fax: 1-732-210-0262 Email: order@genscript.com Web: www.genscript.com

1. Sodium hydroxide, 0.1 N dissolved in LAL Reagent Water, for pH adjustment.
2. Hydrochloric acid, 0.1 N dissolved in LAL Reagent Water, for pH adjustment.
3. Oven or non-circulating hot water bath (37 ± 1°C)
4. Test tube rack.
5. Vortexer.

IV STORAGE

The kit can be stored dry at room temperature for up to one month. For long-term use, the kit can be kept at 2–8°C for up to one year. Do not freeze the kit or any of its components.

V ENDOTOXIN DETECTION PROTOCOL

1. Specimen Preparation

All materials or diluents used for specimen collection and test reagent preparation must be endotoxin-free. Use aseptic technique at all times. Samples to be tested must be stored in such a way that all bacteriological activity is stopped or the endotoxin level may increase over time. For example, samples can be stored at 2-8 ℃ within 24 hours before use, but need to be stored frozen if not used within 24 hours.

Since the LAL-endotoxin reaction is pH dependent, it may be necessary to adjust the pH of the sample to within the range 6.0-8.0 using endotoxin-free sodium hydroxide or hydrochloric acid. Always measure the pH of an aliquot of the bulk sample to avoid contamination by the pH electrode.

Dissolve or dilute test specimen using LAL Reagent Water. It may be necessary to determine the degree of dilution by calculating the MVD value. Maximum Valid Dilution (MVD) is equal to the expected maximum endotoxin concentration in the test sample divided by lambda. Lambda is labeled lysate sensitivity of endotoxin standard. In this kit, lambda is 0.25 EU/ml.

Note: The specimen should be certified free of Beta Glucans contaminant which may come from yeast and cellulosic materials, such as blood products.

2. Reagent Preparation

Limulus Amebocyte Lysate (LAL)

Reconstitute lyophilized lysate by adding 2 ml LAL Reagent Water to the vial. Swirl gently for at least 30 seconds to thoroughly dissolve the lysate. Do not shake or vortex to avoid foaming. Reconstituted lysate can be stored at -20°C or below for up to one week if frozen immediately after reconstitution. Avoid repeated

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freeze and thaw cycles.

***E. coli* Endotoxin Standard**

Reconstitute *E. coli* Endotoxin Standard to 0.5 EU/ml by adding 1 ml LAL Reagent Water to the vial. Mix thoroughly for at least 15 minutes with a vortexer to obtain an endotoxin stock solution. Reconstituted endotoxin standard can be stored at -20°C or below for up to 15 days.

3. Test Procedure

Each assay should include both a positive control and a negative control. LAL Reagent Water can be used as a negative control.

1) Carefully dispense 0.1 ml of LAL reagent into the endotoxin-free vials. Label them as negative control, positive control and sample, respectively.
2) Carefully transfer 0.1 ml of positive control, negative control and the test samples to the LAL reagent in step (1). Cap the vials and mix them thoroughly.
3) Place all the vials in the incubation rack and incubate the vials at 37±1°C by placing the rack in a non-circulating hot water or oven.
4) Remove the rack after 60 ± 2 minutes of incubation, invert each vial and check whether a gel is formed or not.
   a) A positive reaction is characterized by the formation of a firm gel that remains intact when the vial is inverted.
   b) A negative reaction is characterized by the absence of a solid clot. The lysate may show an increased turbidity or viscosity. This is considered a negative result.
5) Calculation of endotoxin level. In this test, the endotoxin level in the positive sample is equal to or higher than 0.25 EU/ml; while in the negative sample, the endotoxin level is lower than 0.25 EU/ml.

4. Application Example

1) Sample: Protein A (1 mg/ml in PBS, pH 7.4) purified from recombinant *E. coli* lysate using Ni-NTA Resin.
2) Dilutions using LAL Reagent Water: 1: 200,000, 1: 400,000, 1: 800,000.
   The test is performed as the procedure above and the assay result is shown in the table below,

Positive control	Negative control	1: 200,000	1: 400,000	1: 800,000
+	-	+	-	-

3) Endotoxin concentration in this sample is, therefore, between 50,000 and 100,000 EU/ml.

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Tel: 1-732-885-9188 Fax: 1-732-210-0262 Email: order@genscript.com Web: www.genscript.com

VI ORDERING INFORMATION

Product Name	Cat. No.
ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (32 rxns)	L00350
ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (16 rxns)	L00350C
ToxinSensor™ Gel Clot Endotoxin Assay kit	L00351
ToxinEraser™ Endotoxin Removal kit	L00338

For *In Vitro* Research Use Only.

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