INHIBITORY EFFECT OF ESSENTIAL OILS AGAINST ORAL PATHOGENS AFFECTING TITANIUM-BASED DENTAL IMPLANTS

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DECLARATION OF INDEPENDENT WORK

I, Sinazo Sibusisiwe Ntondini, student number _____, hereby declare that this research submitted to the Central University of Technology, Free State, for the degree Master of Health Science: Environmental Health is my own original work. I declare that this research has not been submitted previously for the qualification purposes and that it is my own work. The evidence contained is my own knowledge, understanding, the work and ideas of others are clearly cited and referenced.

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Ntondini Sinazo Sibusisiwe **Date**

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SUMMARY

Periodontal diseases, that can lead to tooth damage, affect more than half of the adult population globally. Such diseases are mainly caused by bacterial infection that contaminates the surface of the teeth. Oral illnesses are introduced by bacterial contamination in oral cavities and these bacteria incendiary responses that will continue unless the source of contamination is eliminated through dental treatment. The development of dental caries involves Gram-positive bacteria producing acids as a by-product of the metabolism of fermentable carbohydrates, such as *Streptococcus mutans, Lactobacillus spp* and *Actinomyces*.

The food we eat can be infested with oral pathogens that can cause tooth damage and rehabilitation generally requires the insertion of implant material. Biomaterials utilised for implant manufacturing are not themselves antibacterial agents, thus their surfaces need to be coated with antimicrobial agents to prevent bacterial colonisation of the implant surface. Titanium and its alloys are the most frequently used dental implant materials to replace a missing tooth and have been used for a long time. The most preferred are pure titanium (cpTi) and Ti6Al4V, which give clinical success rates of up to 99% over a period of 10 years. Both these biometals are biocompatible and are capable of undergoing osseointegration. Titanium alloys are widely used in medical applications because they demonstrate excellent biocompatibility and good mechanical properties, such as less elastic modulus than stainless steel or CoCr alloys. However, although titanium alloys are good biomaterials, they can fail due to microbial colonization on the surface of the implant which causes infections and thus their surfaces must be modified to prevent implant infections. The current research focused on using natural products, particularly plant-derived essential oils, to combat bacteria that colonise dental implants and later cause implant failure. The literature has revealed that natural products such as essential oils are promising antimicrobial agents.

Rising knowledge about the emergence of antibiotic-resistant microorganisms has encouraged researchers to search for new antimicrobial agents that are more effective

against resistant microbial pathogens than currently preferred products. It was against this background that the current study investigated the antibacterial activity of five commercially available plant-derived essential oils, namely *Lavandula latifolia* (lavender oil), *Syzygium aromaticum* (clove bud), *Salvia officinalis* (sage), *Cinnamomum zeylanicum Blume* (cinnamon) and *Mentha piperita* (peppermint). These essential oils were tested against three resistant pathogens, namely *Staphylococcus aureus, Streptococcus mutans* and *Escherichia coli.*

The chemical components of the essential oils were analysed using gas chromatography-mass spectrometry (GC-MS) and the main components of the essential oils were found to be terpenes and phenols. The antimicrobial activity of the essential oils was additionally investigated using agar diffusion bioassay from five essential oils. Of these, *Cinnamomum zeylanicum Blume* showed the highest antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus,* whereas *Salvia officinalis* showed the highest antimicrobial activity against *Streptococcus mutans*. The minimum concentrations at which the essential oils inhibited bacterial growth were investigated using microdilution essay. Structural changes caused by the essential oils were evaluated using a scanning electron microscope and alterations such as damaged cell walls, holes in the bacterial cells, irregularity in size and some ruptured cells were observed. After scanning electron microscopy had been performed, Ti6Al4V (ELI) experimental dental implants (which are used to replace a missing tooth) were additively manufactured (3D printing) using EOS M280 direct metal laser sintering (DMLS) manufacturing technology. A subset of the laser powder bed fusion (LPBF) process was also employed. Next, the antimicrobial activity of the essential oils was investigated on the surface of the titanium implant materials. The essential oils were used to modify the surface of titanium implant materials to inhibit the growth of bacteria on their surfaces.

The findings suggest that antimicrobial agents such as essential oils need to be considered as potential antimicrobials in the future because of their effective mechanism of action against bacterial cells. For instance, it was found that *Cinnamomum zeylanicum Blume* and *Salvia officinalis* essential oils penetrated the bacterial cell wall and gained

entrance into the cell, thus causing disruption to the entire bacterial cell. Essential oils thus have the potential to be used as antimicrobial agents against *Staphylococcus aureus, Streptococcus mutans* and *Escherichia coli*. Scanning electron microscopy (SEM) results also showed radical structural alterations such as leakage of bacteria cell contents, cells becoming pleomorphic, irregularity in cell size and the rupturing of some cells when treated with *Salvia officinalis* and *Cinnamomum zeylanicum Blume* essential oils at their minimum inhibition concentrations. The bioassay results showed that *Lavendula officianalis*, *Mentha piperita, Cinnamomum zeylanicum Blume and Salvia officinalis* essential oils can act as effective antimicrobial agents against *Staphylococcus aureus, Streptococcus mutans* and *Escherichia coli*. It is noteworthy that *Salvia officinalis* and *Cinnamomum zeylanicum Blume* essential oils showed the most significant inhibitory effects on oral pathogens in the present work.

Central University of Technology, Free State

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PUBLICATIONS EMANATING FROM THIS STUDY

1. **Ntondini S.S.**, Lenetha G.G. and Dzogbewu T.C. 2021. Inhibitory effect of essential oils against oral pathogens. *International Journal of Dentistry and Oral Science*, 8(1):1308-1313. doi: dx.doi.org/10.19070/2377-8075-21000259

2. **Ntondini S.S.**, Lenetha G.G. and Dzogbewu T.C. 2021. Antimicrobial activity of *Salvia Officinalis* against *Streptococcus mutans* causing dental implant failure. *Journal of International Oral Health*. (Accepted).

CHAPTER ONE

BACKGROUND

1.1 Introduction

1.1.1 Oral health

The prevalence of oral diseases is a worldwide public health problem that affects over 3.5 billion people. According to the World Bank, this has negative economic and health impacts, particularly in low-income countries (Beaglehole, 2019; Peres et al., 2019; Watt et al., 2019). In many low-income countries such as Central African Republic, Benin, Burundi, Burkina Faso, Ethiopia, Lesotho, Mali, Malawi, Mozambique and Uganda, oral diseases continue to be untreated because the treatment costs exceed available resources (Fantom and Serajuddin, 2016; Bist, 2018; Peres et al., 2019). Oral diseases remain a major public health problem in South Africa and this issue needs to be addressed urgently − particularly in disadvantaged communities (Singh, 2011; Bhayat and Chikte, 2018). Approximately 500 bacteria species are found in the oral cavity and they are responsible for colonising different exteriors such as prosthetic devices and dental implants (Liu et al., 2016). Amongst these bacteria species are *Porphyromonas gingivalis* and *Streptococcus mutans,* which are two of the most significant species as they are responsible for encouraging biofilm development, inflammatory response and bone defects (Liu et al., 2016).

Exceedingly prevalent and noteworthy oral diseases worldwide are dental caries that are commonly identified as tooth decay, periodontal disease, tooth loss and cancer of the lips (Ali and Al-dahan, 2019). Tooth decay continues to pose a significant public health problem across the world (WHO, 2016). The World Health Organization (WHO) highlights that such infections affect about 60–90% of school children, while in adults it contributes to extensive loss of natural teeth globally (WHO, 2016; Petersen and Ogawa, 2016). Mathur and Dhillon (2018) also believe that tooth decay is one of the most prevalent diseases, arguing that it affects about 50% of children across the globe.

Tooth decay is an infectious disease process that causes damage to the structure of the tooth (Caruso et al., 2016; Ali and Al-dahan, 2019). Tooth decay occurs as the localised damage of hard dental tissue by acidic by-products from bacterial fermentation of dietary carbohydrates. The major cause of tooth decay is the bacterial biofilm that covers the tooth surface (Selwitz et al., 2007; Pitts et al., 2017). This biofilm development is mostly sugar motivated and results in the phasic demineralisation and remineralisation of hard dental tissue (Pitts et al., 2017). Tooth decay arises primarily on the tooth crown and later affects the root surfaces if it is not treated.

According to Moztarzadeh (2017), teeth are very important to all people for chewing, speech and aesthetics. Bacterial development in the mouth causes an individual to lose a tooth (or more) and therefore dental implantology was technologically advanced to create dental implants to replace a lost tooth or teeth (Moztarzadeh, 2017). More recently, titanium implants have become the product of choice for this purpose. A lost tooth is replaced with dental implants to which a crown, bridge or denture can be affixed. However, endosseous implants can fail either at the initial or later stage of insertion (Esposito et al., 2003; Esposito et al., 2013). This may be caused by periodontitis and peri-implantitis, which are inflammatory diseases caused by periodontal pathogenic bacteria resulting in the destruction of the supporting peri-implant tissue. As the bacteria drift down the surface of the tooth or the titanium implant, the inflammation extends along with it (Aas et al., 2008; Esposito et al., 2013).

1.1.2 Oral pathogens found in the mouth

Oral pathogens' method of transmission, such as by means of *Staphylococcus aureus* and *Escherichia coli,* can occur through the digestion of polluted food or water (Chao et al., 2017). *Escherichia coli* is a nosocomial pathogen generally known to cause urinary tract infections (Predoi et al., 2018). *Escherichia coli* can also cause diarrhoea if contaminated food is consumed or if foul water is drunk and it is thus commonly associated with food poisoning (Chao et al., 2017). *Escherichia coli* produces a toxin called *shiga and* this toxin damages the lining of the intestine (Chao et al., 2017).

According to Ardila and Villa-Correa (2015), *E. coli* is related to initial implant failure as this bacterium is resistant to antibiotics such as doxycycline, amoxicillin, metronidazole and aminoglycosides (Ardila and Villa-Correa, 2015). Early implant failure is also associated with certain strains of bacteria such as *Streptococci*, anaerobic Gram-positive cocci and anaerobic Gram-negative rods (Ahmad and Saad, 2012; Hwang et al., 2021). A major health problem is that bacteria introduced during the placement of implants can lead to infection and, in a worst-case scenario, to implant failure (Esposito et al., 2003; Esposito et al., 2013; Sridhar et al., 2015). The bacteria are mainly found in dental plaque that forms on the surface of dental implants. The primary bacterial colonizers on the surface of dental implants are *Streptococci* (*Streptococcus viridans, Streptococcus mitis* and *Streptococcus oralis)*, *Porphyromonas gingivalis, and Prevotella intermedia* and *Streptococcus sobrinus* (Leonhardt et al., 1999; Dhir, 2013). Secondary colonizers are predominantly the *Actinomyces* species and *Streptococcus mutans* (Leonhardt et al., 1999; Dhir, 2013).

According to Sridhar et al. (2015), bacterial colonization on the surface of dental implants is the main contributor to the increasing number of dental implant failures and thus preventative treatment options are needed. However, infections around biomaterials such as dental implants are hard to treat and nearly all infected implants must be removed, which is why it is crucial to avoid infection (Esposito et al., 2003; Esposito et al., 2013).

1.1.3 Oral health preventative and treatment techniques

Oral health education, especially at an early age, can be a powerful technique to prevent chronic diseases such as dental caries (Umeda et al., 2020). Preventative techniques of tooth decay can include brushing and flossing daily, the use of antimicrobial mouth rinses, fluoride treatment, seeing a dentist for routine care and good nutrition to maintain the overall health of the teeth (Phinney and Halstead, 2017).

Medication options such as prophylactic systemic antibiotic regimens have been proposed to minimise infections after dental implant placement. However, adverse health effects may arise with the administration of antibiotics and can range from diarrhoea to life-threatening allergic reactions (Esposito et al., 2003). Another major concern associated with the extensive use of antibiotics is that bacteria have developed antibiotic resistance due to the over prescription of antibiotics for individuals (Dhir, 2013). The usage of prophylactic antibiotics in implant dentistry is therefore controversial. The binge of drug-resistant pathogens is a major public health problem that has prompted research into the identification of new biocides with broad antimicrobial activity (Oussalah et al., 2006; Nazzaro et al., 2013). Thus, the application of alternative medicines, such as the use of essential oils, has come to the forefront in treatment regimens because of their antimicrobial activity. Such treatments are also preferred as recent developments in medicine and biotechnology have not been able to overcome the rapid and continuing worldwide emergence and spread of drug resistant microorganisms (Predoi et al., 2018). This has encouraged the search for new types of effective and nontoxic antimicrobial agents among natural compounds that are found in aromatic plants and that are still used in folk medicine, cosmetics and aromatherapy. It is projected that approximately 700 000 species of tropical plants show medicinal properties and can be used as antibacterial, antifungal, antiallergic and anticarcinogenic agents (Predoi et al., 2018). Essential oils in particular, but also other plant extracts, have aroused interest as sources of natural protection against pathogens (Thosar et al., 2013; Fani and Kohanteb, 2017). This increased interest in the use of natural products such as essential oils has been awakened because of the secondary metabolites produced by various medicinal plants that have antibacterial, antifungal and antioxidant properties (Ardila and Villa-Correa, 2015; Wawrzyńczak et al., 2021).

Research have been conducted to demonstrate the therapeutic properties of various essential oils such as tea tree oil, clove oil, peppermint oil, cinnamon oil and sage oil (Ardila and Villa-Correa, 2015; Wawrzyńczak et al., 2021). However, there is a dearth of publications on the use of essential oils in the dentistry field.

These essential oils are natural oils extracted from plant materials such as buds, flower petals, twigs, leaves, roots and seeds (Oussalah et al., 2006; Thosar et al., 2013; Wawrzyńczak et al., 2021). The essential oils are isolated from the plant materials by using the hydro or steam distillation methods (Irshad et al., 2020). The current intense research into the use of essential oils for dental implant infection control has been triggered by the knowledge that essential oils may inhibit the growth of drug-resistant microbial strains that are difficult to treat even by means of conventional antibiotics (Tariq et al., 2019, Nazzaro et al., 2013). However, there is still limited development regarding the effects of essential oils on the health and well-being of the oral cavity.

It is for the reasons discussed above that this study focused mainly on the antimicrobial properties of essential oils to determine the extent to which they may prevent oral pathogens from affecting dental implants. More specifically, the inhibitory effects of lavender, sage, peppermint, and cinnamon and clove essential oils were tested against *S. aureus, S. mutans* and *E. coli* that are mainly responsible for dental implant failure.

1.2 Rationale for the Study

According to the World Health Organization (2014), regardless of excessive advancements in oral health in several countries, oral problems continue, particularly among unprivileged groups of people in both developing and developed countries, including South Africa (Dagli et al., 2015). Dental caries and periodontal diseases are persistent oral health problems globally as they adversely affect the quality of life and working capability of individuals (Dagli et al., 2015).

According to Moztarzadeh (2017), teeth are very important to all people for chewing, speech and aesthetics. However, it is common that people lose their teeth due to tooth decay that is caused by bacterial growth in the mouth. Therefore, dental implantology was introduced as a form of treatment for this phenomenon (Moztarzadeh, 2017). Dental

implants are used to reinstate lost teeth and are generally preferred above other options (Moztarzadeh, 2017).

However, dental implant failure is a major problem that is caused by bacterial contamination at the surface of implants. Moreover, infections around the surface of implants are difficult to treat and the worst-case scenario is having to remove the implant. Antibiotics are used for infection control, but the use of antibiotics in implant dentistry is controversial due to antibiotic resistance (Chouirfa et al., 2019). It is therefore important to determine whether prophylactic antibiotics are effective in avoiding dental implant failure as there is wide concern regarding the widespread use of antibiotics and their diminishing effectiveness on antibiotic resistant bacteria (Esposito et al., 2013).

Peri-implant colonization with pathogenic microorganisms is a major cause of oral infections (Pedrazzi et al., 2014). Inflammation is normally caused by a biofilm that forms around the surface of the implant. The biofilm invasion causes an immune inflammatory reaction in local tissue and may lead to inflammatory processes in gums which is known as gingivitis or peri-implant mucositis (Pedrazzi et al., 2014). Saini (2011) agrees that biofilm development on oral implants can cause inflammation of peri-implant tissue, which jeopardises the long-term success of dental implants. The formation of a biofilm plays a significant role in the spread of antibiotic-resistant bacteria.

Khalil et al. (2016) confirm that antibiotics are used to slay bacteria that can cause infection and disease. It is undeniable that antibiotics have made a major contribution to human health; however, some bacteria have developed resistance to commonly used antibiotics such as amoxicillin, methicillin and metronidazole (Nigam et al., 2014; Khalil et al., 2016). Moreover, some bacteria such as *S. aureus* can survive and even reproduce in the presence of antibiotics (Nigam et al., 2014; Khalil et al., 2016).
Dantes et al. (2013) state that infections allied with methicillin-resistant *Staphylococcus aureus* (MRSA) present a substantial problem to clinicians as *S. aureus* infections are related to elevated morbidity and mortality rates.

This study thus focused on using naturally based products such as essential oils to control pathogens that affect dental implants.

1.3 Aim and Objectives of the Study

This study aimed to examine the antimicrobial activity and efficacy of essential oils against oral pathogens that affect titanium dental implants.

The objectives of the study were to:

- investigate the chemical composition of selected essential oils using gas chromatography-mass spectrometry (GC-MS);
- confirm whether the selected essential oils could inhibit oral bacteria affecting titanium dental implants by using bioassay method;
- investigate minimum inhibition concentrations (MICs) of the selected essential oils against oral pathogens using microdilution assay;
- investigate the structural changes caused by essential oils on bacteria cells using scanning electron microscopy (SEM) and transmission electron microscopy (TEM);
- Confirm whether essential oils have antimicrobial activity against oral pathogens when plated on the surface of titanium implant materials (i.e., by coating implant materials with selected essential oils).

A diagram that illustrates the objectives of the study is presented in Figure 1.1.

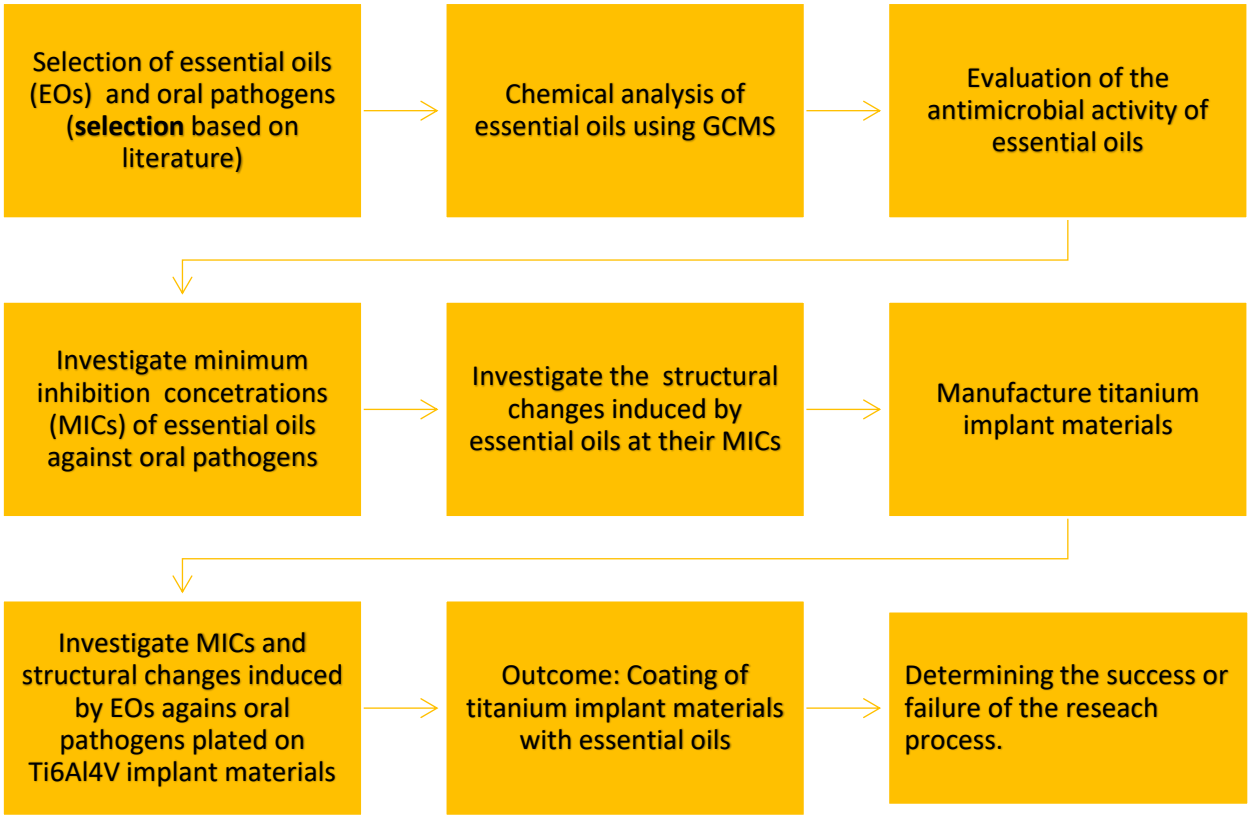

Figure 1.1: Diagrammatic summary of the objectives of the study

1.4 Chapter Summary

The prevalence of bacterial biofilm infections continues to be a reason for dental implant failure. The development of a bacterial biofilm on titanium dental implant material surfaces may lead to the development of peri-implant diseases that influence the long-term success of dental implants if not treated. Various biomaterials are used in dentistry, of which titanium is considered the most biocompatible. However, without the application of appropriate antimicrobial agents, titanium and its alloys (i.e., biomaterials) cannot prevent implant failure due to bacterial colonization on the implant surface. Therefore, long-term clinical success becomes dependent on antimicrobial properties. The prevention of biofilm development on biomaterial surfaces is the best way to avoid both the spread of pathogens and biomaterial deterioration. Essential oils are promising antimicrobial agents that could be used to prevent biofilm development on the surface of biomaterials such as titanium implants to prevent dental infection. The emergence and spread of drug resistant microorganisms continue to be a public health problem, thus the current study focused on the use of natural products, particularly plant-derived essential oils, to combat antibiotic-resistant microorganisms in dental cavies.

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CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

The chief benefit of natural products such as essential oils, is that they do not increase antibiotic resistance compared to the long-term use of synthetic antibiotics (Högberg et al., 2010; Fournomiti et al., 2015). According to Kavanaugh and Ribbeck (2012), plant-derived essential oils have been used for hundreds of years as natural medicines to contest a wide range of pathogens, including bacteria, fungi and viruses. Furthermore, in recent years there has been an increased interest in the use of essential oils because of their antifungal, antibacterial and antioxidant properties (Dagli et al., 2014; Pandey et al., 2017). Moreover, apart from their antibacterial properties, essential oils have been described to show antiviral, antimycotic, antioxygenic, antiparasitic and insecticidal properties (Predoi et al., 2018).

Medicinal plants that contain essential oils have been used in developing countries as complementary treatments for health problems (Bernardes et al., 2010; Manion et al., 2017). There is thus an escalating interest in what is termed ‘green consumerism’ that has led to the use of plant-derived natural products. These products are used as preservatives and essential oils and their compounds are acknowledged to possess inherent antimicrobial properties (Dagli et al., 2015; Prakash et al., 2018). Essential oils are also used in different industries. For instance, they are used in perfumes and lotions, in foodstuff as preservatives and for additives and in pharmaceutical products for therapeutic action (Zyglo and Julian, 2000; Prakash et al., 2018). There has thus been an increasing inclination towards the use of organic and natural products among health-conscious consumers. For instance, the global essential oils market was reported to be 226.8 kilotons in 2018and it is expected to increase to a compound annual growth rate (GAGR) of 8.6% from 2019 to 2025 (Irshad et al., 2020).

2.2 Health Benefits of Essential Oils

Essential oils were globally used in the past to treat various infectious diseases and this interest has been rekindled in the modern era. For example, essential oils are used in beverages, food industries, for personal care, in cosmetics, in insecticides against different pests and in the treatment of dermatological issues (Irshad et al., 2020). De Rapper et al. (2016) note that essential oils provide many health benefits when used properly and that one of their major uses is in oral health care (Table 2.1).

2.3 Mode of Action of Essential Oils against Bacteria

Bacteria have a cell membrane that serves as a blockade between the cytoplasm and the external environment. Gram-positive bacteria consist of a tough and rigid mesh cell wall, whereas Gram-negative bacteria have a thin cell wall surrounded by an outer membrane that is an additional protective layer (Kapoor et al., 2017). The cell membrane remains important for the existence of bacteria, whereas the cytoplasm exists to prevent ions from flowing into and out of the cells of bacteria. When bacteria are treated with antimicrobial agents such as essential oils, these oils degrade the cell membrane which is detrimental to the cytoplasmic membrane. This causes cytoplasmic coagulation, thus damaging the membrane proteins and increasing penetrability, which leads to the leaking of cell substances and its subsequent destruction (Nazzaro et al., 2013; Chouhan et al., 2017). The cytoplasmic membrane of a bacterium cell has a pump function that takes antimicrobial agents out of the cell, which is referred to as the efflux pump mechanism. However, essential oils degrade the entire cell, thus making it difficult for bacteria to fight against them (Nazzaro et al., 2013; Chouhan et al., 2017; Yang et al., 2020). It is known that essential oils and their components act upon a variety of targets, such as the cell membrane and cytoplasm. In some cases, they totally change the structure of the bacterial cells (Nazzaro et al., 2013; Swamy et al., 2016). However, the chemical components of plants' essential oils are different and these differences are linked to the nature of their antimicrobial activities against diverse pathogenic microorganisms (Voon

et al., 2012; Swamy et al., 2016). Commonly, however, these essential oils have a hydrophobic nature, which is what allows them to penetrate microbial cells.

Table 2.1: Health benefits of essential oils

Essential oil	Composition	Pathogens	Applications	References
***Eucalyptus globulus* (Eucalyptus oil)**	1,8-cineole followed by cryptone, α-pinene, *p*-cymene, α-terpineol, trans-pinocarveol, phellandral, cuminal, globulol, limonene, aromadendrene, spathulenol and terpinene-4-ol	*Escherichia coli, Staphylococcus aureus*	Anti-cariogenic agent Antiseptic for the treatment of measles, scarlet fever, influenza and typhoid fever	(Irshad et al., 2020; Gherasim et al., 2021).
***Mentha piperita* (peppermint oil**	Menthol (major compound), menthyl acetate and menthofuran	*Staphylococci, Enterococcus fecalis, Staphylococcus aureus, Candida albicans and Escherichia coli*	Antimicrobial activity - Utilised in oral hygiene products, for example in mouth rinses. Remarkably powerful for combating oral pathogens and killing common bacteria that cause cavities and gum disease. Is also used to freshen	(Irshad et al., 2020).

			your breath. Also, for the treatment of Exhaustion, nausea, fever and asthma	
***Melaleuca alternifolia* (Tea Tree Oil)**	terpinen-4-ol, γ-terpinene, *p*-cymene, α-terpinene, 1,8-cineole, α-terpineol and α-pinene	*C.albicans, Staphylococcus*	Oral candidiasis - Suitable for use in prophylactic oral hygiene products. Antifungal activity: *Melaleuca alternifolia* comprises antimycotic activity, terpinen-4-ol being its most active component Used to treat cold sores. Also, antibacterial and antifungal for cuts and flu	(Warnke et al., 2009; Irshad et al., 2020).
***Syzygium aromaticum* (Clove oil)**	phenylpropanoids eugenol, eugenyl acetate, carvacrol, thymol, cinnamaldehyde, β-caryophyllene and 2-heptanone	*C.albicans, Staphylococcus*	Natural ability to control the develop-ment of bacteria and can help fight mouth and throat infections	(Warnke et al., 2009).
***Lavendula officinalis* (lavender oil)**	50% linalyl and 35% linalool of linalyl acetate (3, 7-dimethyl-1,	*Enterococcus fecalis, Staphylococcus aureus, Candida*	Used as an anxiolytic in dental clinics. Reduces pain of needle insertion. Treating painful	(Dagli, 2014; De Rapper et al., 2016).

	6 octadien-3yl acetate), linalool (3, 7-dimethylocta-1, 6-dien-3-ol), lavandulol, 1, 8-cineole, lavandulyl acetate	*albicans* and *Escherichia coli*	inflammation of the skin. It is also used in wound healing processes as this oil promotes healing of the skin tissue	
***Leptospermum scoparium* (Manuka oil)**	caryophyllene, geranoil, pinene, humulene, linaloool and leptospermone (Waenke et al., 2009)	*Enterococcus fecalis, Staphylococcus aureus, Candida albicans* and *Escherichia coli*	This oil helps the scars and after marks on the skin to fade away by promoting new cell growth in the affected parts of the body and protecting wounds from developing infection	*(*Warnke et al., 2009; (Patil, 2019)

It also causes a change in the structure and function of Gram-positive bacteria (Chouhan et al., 2017). Essential oils are regarded as rich in phenolics that enable them to penetrate the phospholipids bilayer of the bacteria cell wall and to bind with proteins. This prevents bacteria cells from executing their normal functions (Nazzaro et al., 2013; Chouhan et al., 2017).

2.4 Antibiotic Resistance

The growing resistance of microorganisms to conventional chemicals and drugs is continuing to be a global problem that has driven research into finding new biocides with

far-reaching action (Nazzaro et al., 2013; Alcock et al., 2020). One of the main causes associated with antibacterial drug resistance is the misuse and overconsumption of these drugs that are found in human medicine and in agricultural products (Chokshi et al., 2019). The food chain is also considered a main route of transmission of antibiotic resistant bacteria among animal and human populations. Because the antibiotics that are used in agriculture often have the same or similar compounds used clinically for the treatment of various infections, this overuse can contribute to drug resistance (Zaman et al., 2017). The World Health Organization (2016) states that antibiotics are largely used to combat bacteria that can cause illnesses, diseases and infections, but antibiotic resistance remains a public health problem. In the earlier years of antibiotic use, they had a major impact on extended human health as many diseases that had once caused death were effectively treated with antibiotics. However, some bacteria such as *Staphylococcus aureus* have become resistant to commonly used methicillin, amoxicillin and metronidazole antibiotics and *Escherichia coli* have become resistant to ciprofloxacin (World Health Organization, 2016; Moradigaravand et al., 2018).

Antibiotics can commonly get rid of most bacteria in a colony, but a different strain of bacteria that has mutated genetically can result in resistance (Zaman et al., 2017). Antibiotics also fail to inhibit bacterial growth because bacteria change or limit the number of openings in the cell wall. Antibiotic drug molecules normally gain access to the cell by diffusion through the porins found in the outer membrane of Gram-positive bacteria, which means that a reduction in the number of porin channels leads to a decrease in the entry of antibiotics into the cell (Nazzaro et al., 2013; Kapoor et al., 2017). The cytoplasmic membrane of bacteria has pumps that take antibiotics out of the cell and these ‘pumps’ are known as efflux mechanism pumps. For example, Tetracyclines, Macrolides, Lincosamides, Streptogramins, Oxazolidinones, Phenicols, Cationic peptides, Lipopeptides, Quinolones, Pyrimidines, Rifamycins and the Sulfonamides groups of antibiotics are pumped out in this manner (Zaman et al., 2017). The pumps function at the same speed as the speed at which antibiotics gain access to the cells and they thus pump the antibiotic out before it reaches the target.

The outer membrane of a Gram-positive bacterium thus keeps the antibiotic from gaining entrance into the cell (Nazzaro et al., 2013; Kapoor et al., 2017).

It is for this reason that microorganisms are becoming more antibiotic resistant as they can survive and increase in the presence of antibiotics. They tend to prevent the antimicrobial from meeting its target by limiting its ability to enter the cell. Essential oils, on the other hand, have shown great potential in the biomedicine field as they have the ability to efficiently destroy several fungal and viral pathogens. Essential oils inhibit the growth of pathogens by targeting the membrane and cytoplasm and, in some cases, they totally alter the structure of the cells (Warnke et al., 2009; Chouhan et al., 2017).

In the dentistry field, essential oils have mainly been utilised to provide calmness and relaxation to patients and to manage emotional distress. Aromatherapy is also regarded as useful in treating oral ulcers and toothache (Baig et al., 2017). However, it has been argued that more intensive research needs to be conducted on the broader usage of essential oils in dentistry (Ferreira et al., 2021) which was the focus of the current study.

2.5 Dental Caries

The term dental caries is defined as the destruction of vulnerable hard dental tissue by acidic by-products that are released from the bacterial fermentation of dietary carbohydrates. The major cause of dental caries is the bacterial biofilm that shelters a tooth surface (Selwitz et al., 2007; Pitts et al., 2017). This biofilm development is generally sugar driven and results in the phasic demineralisation and remineralisation of hard dental tissue (Pitts et al., 2017). Dental caries occurs primarily upon the tooth crown but later also affect the root surfaces of teeth if not treated.

Figure 2.1 shows the anatomy of a normal tooth. Bacteria colonise the hard tissue of a tooth, which is the enamel. The hard tissue of the tooth is comprised of enamel, dentine and cementum. Enamel is a hard material containing mainly hydroxyapatite and shelters the dentine on the crown of the tooth. Cementum is a bone-matrix-like substance that is

composed of mineral and collagen and encompasses the root of the tooth. The dental pulp shapes the central part and is comprised of connective tissue, blood vessels and nerves. Teeth are enclosed by a salivary pellicle layer that contains proteins and glycoproteins that facilitate attaching of the oral microbiota to the teeth. This structure is called the dental biofilm, also known as dental plaque. The biofilm closes off the surface enamel from the saliva and oral cavity and produces a protected microenvironment at the tooth surface. Gingiva, which is commonly known as the gum, surrounds the teeth (Pitts et al., 2017). Saliva, which is a specialised fluid that is secreted in the mouth throughout the day, maintains the integrity of the teeth. Saliva in the mouth is vital as it combats oral pathogens by buffering the oral environment at a neutral pH, which is ideal for the development and metabolism of most of the oral pathogens, while also supplying proteins and glycoproteins as nutrients. Biofilm development that causes dental caries normally occurs at night when there is no movement of the saliva that protects the mouth and teeth against bacteria (Petersen and Ogawa, 2016; Pitts et al., 2017).

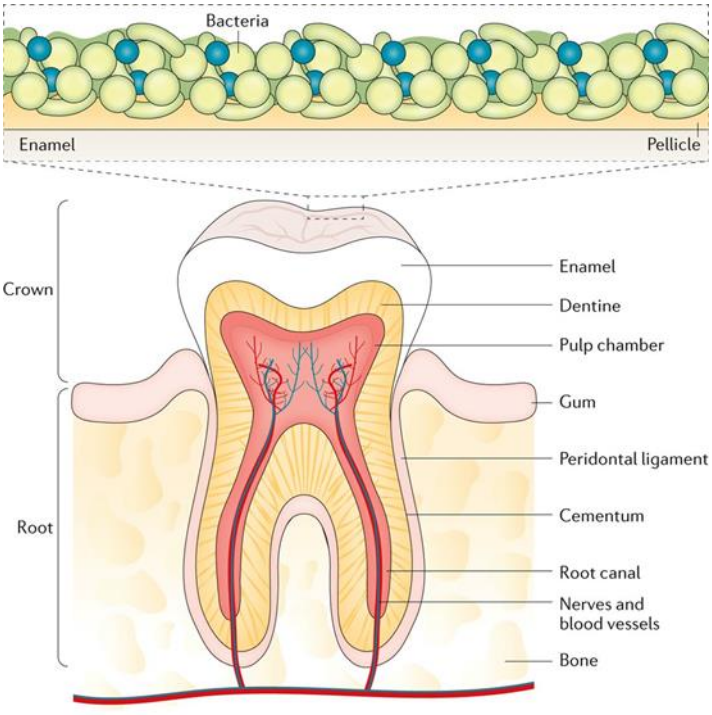

Figure 2.1: Composition of a tooth

Source: Pitts et al., 2017

Dental caries pose a substantial public health challenge across the world. Dental caries affect about 60–90% of school children and a large majority of adults as they contribute to a massive loss of natural teeth globally (Petersen and Ogawa, 2016; WHO, 2016). It is for this reason that the study focused mainly on the avoidance of oral pathogens that affect dental implants when a tooth was (or teeth were) lost.

2.6 Dental Implants

A dental implant is normally a small screw finished with a biometal such as titanium which is biocompatible with the tissue surrounding the tooth. It is not harmful or toxic to living tissue (Coffigniez et al., 2021). The implant is inserted into the jawbone to replace the missing tooth (Meyerov et al., 2012). Titanium and its alloys are the backbone material for producing such biomedical devices in the dental field due to their distinctive combination of chemical, physical and biological properties. The preference for Ti-based alloys for biomedical applications above other biometals is due to the development of a very thin adherent protective titanium that is a stable oxide film in an oxidizing environment which spontaneously contributes to the passivation or repassivation procedure to prevent corrosion and promote biocompatibility of the metal. Ti6Al4V is the most used titanium alloy (Gepreel and Niinomi, 2013; Moztarzadeh, 2017). Aluminium increases the strength and decreases the alloy weight while vanadium decreases the alloy proneness to corrosion (Moztarzadeh, 2017). According to the periodic table, titanium consists of 47 890 atomic weight, 22 atomic number 4.3 oxidation state, 3562 k boiling point and 1943 k melting point. Titanium alloys have largely been used since 1981 to replace a missing tooth (Abdulgader, 2016; Nicholson, 2020). The most important alloys are pure titanium (cpTi) and Ti-6Al-4V, both of which have provided clinical success rates of up to 99% in a 10-year period (Abdulgader, 2016; Nicholson, 2020).

Zhao et al. (2009) and Abdulgader (2016) note that, although titanium alloys are good materials, they can fail due to microbial colonisation on the surface of the implant which causes infection. This statement is based on the premise that the same surface properties that make Ti-based implants biocompatible are responsible for its surface susceptibility

for bacterial infections. The development of the thin titanium oxide layer under physiological conditions is a good substrate for the adhesion of proteins and cells which enhance osseointegration. However, these physiological conditions that make Ti-based implants the preferred biometal can equally provoke bacterial colonization and biofilm development on Ti-based implant surfaces, hence titanium and its alloys alone are not able to meet every clinical requirement, especially as they cannot prevent infection without appropriate surface modifications (Elias, 2010). Therefore, the study evaluated whether essential oils can inhibit bacterial growth around the surface of the titanium dental implant.

2.7 Peri-Implant Infections

Implant failure can occur right at the beginning or at a later stage. Early implant failure occurs before osseointegration (i.e., the direct and stable anchorage of an implant due to the development of bony tissue around the implant), whereas late failure occurs years or decades after insertion (Cillo, 2020). Implants' surfaces are prone to colonization by pathogenic bacteria that cause peri-implant tissue destruction and implant failure (Meyerov et al., 2012). Osseointegrated dental implants have a long-term success rate of over 90%, but they are endangered by pathogenic bacteria colonization (Chiapinotto et al., 2012), as peri-implant colonization with pathogenic microorganisms causes infection (Pedrazzi et al., 2014). During surgery, implants are prone to bacterial infection from both skin and mucous membranes. Such infection results from a biofilm that forms around the surface of the implant (Chouirfa et al., 2019), which in turn causes dental inflammation (Costerton, 1995; Pedrazzi et al., 2014; Chouirfa et al., 2019). This biofilm is a bacterium formed aggregate that forms in the presence of liquid on hard surfaces. Biofilm infestation causes an immune inflammatory reaction in local tissue and could lead to inflammatory processes in gums known as gingivitis or peri-implant mucositis (Costerton, 1995; Pedrazzi et al., 2014). Peri-implatitis, which is caused by bacterial colonization, is an irreversible inflammation of the soft and hard peri-implant tissues (Cillo, 2020). Gingivitis is the slightest form of periodontal diseases that are caused by a bacteria biofilm that gathers on the teeth adjacent to the gums.

Periodontitis causes loss of connective tissue and bone support and is a major cause of tooth loss (Bruce and Pihlstrom, 2006; Pedrazzi et al., 2014; Cillo, 2020).

2.8 Common Treatment Methods for Biofilm on the Surface of an Implant

Various strategies may be used to avoid infection on titanium implant surfaces such as surface modification and coatings with antibiotics, antimicrobial peptides, inorganic antibacterial metal elements and antibacterial polymers (Chouirfa et al., 2019). Surface modification of implanted devices is an efficient way of lessening the occurrence of implant-related infection. It is a relatively uncomplicated method to modify the interfacial properties of medical devices without disturbing the bulk properties of the material. Surface modification involves using techniques such matrix supported pulsed laser evaporation to alter the biomaterial surface and to prevent the initial attachment of bacteria. Laser photodynamic, or photothermal elimination of the bacteria flora, appears promising, but it is not available to every clinician, so it may be too specialised to be considered a standard treatment (Warnke et al., 2009; Khatoon et al., 2018). Furthermore, biomaterial properties such as surface area, surface roughness, surface energy and hydrophilicity can also improve or reduce protein adsorption and microbial attachment (Khatoon et al., 2018).

Chemical agents appear to curb peri-implant inflammation, but this requires further research. The use of antimicrobial mouth rinses as a chemical method has become common in the last decade to control biofilm development. Mouth rinses have active ingredients that are used for chemical biofilm control and these ingredients usually include bis-biguanide, essential oils and 0.12% chlorhexidine gluconate. However, chlorhexidine gluconate has several undesirable side-effects such as irritation and staining of the teeth and it needs to be prescribed with caution. The most common therapeutic agents discovered in mouth rinses include essential oil components such as thymol, eucalyptol, and menthol and methyl salicylate. The other chemical components are hexetidine, chlorhexidine gluconate, benzalkonium chloride, cettylpyridinium chloride, hydrogen peroxide and sometimes domiphen bromide, fluoride and xylitol which are known to be

responsible for inhibiting bacterial growth (Pedrazzi et al., 2014). However, the long-term use of mouth rinses containing chlorhexidine has led to discoloration of dental restorations and soft tissue, alterations in the sense of taste, mucosal erosions and parotid gland swelling (Pedrazzi et al., 2014). Chlorhexidine reduces 60% biofilm build-up and gingivitis severity in 50−80% of cases. Rinsing the mouth with treatments containing 0.12% chlorhexidine gluconate results in a major decrease in total anaerobes, total aerobes, *Streptococci and Actinomyces*. However, Pedrazzi et al. (2014) found that after both three- and six-month periods, essential oils had delayed biofilm growth in 45−56% of cases and reduced the existing biofilm in 39−48% of cases, whereas a reduction of up to 59% in gingivitis was also observed after their continuous use. Studies have demonstrated that essential oils influence microbial total mass and promote an overall decrease in both biofilm activity and biomass (Pedrazzi et al., 2014).

The simplest and best known oral care hygiene method is the proper use of a toothbrush to maintain oral health and reduce most oral pathogens. However, this method is not effective enough to maintain good oral hygiene as the toothbrush has difficulty in reaching interproximal areas (Pedrazzi et al., 2014). There are several devices such as dental floss or tape available on the market for use in areas a toothbrush cannot reach (Pedrazzi et al., 2014). Electric and electromagnetic fields have also been explored for the treatment of bacterial colonization as it is known that cells are sensitive to electric fields (Khatoon et al., 2018).

However, regardless of a variety of preventative measures, the prevalence of peri-implant diseases is increasing and therefore the discovery of effective prevention methods seems to be crucial for implant recommendation as well as for the advancement of professional training (Chouirfa et al., 2019). Therefore, the greatest possible treatment for biofilm-induced infections is to prevent bacteria colonization at the initial attachment stage in order to prevent any infection from the beginning.

2.9 Chapter summary

Dental caries are a major public health problem across the world. Dental caries re caused by bacterial biofilm that shelters a tooth surface and later causes tooth loss, therefore biomaterials such as titanium dental implants are used to replace a missing tooth. The titanium implant is inserted into the jawbone to replace the missing tooth; however, it can also fail as bacteria that shelters their surface. Essential oils are promising have been used for hundreds of years as natural medicines to fight a wide range of pathogens, including bacteria, fungi and viruses and therefore there has been an increased interest in the use of essential oils because of their antifungal, antibacterial and antioxidant properties. Antibiotic resistance is a major health problem as microorganisms can survive and increase in the presence of antibiotics however the chief benefit of natural such as essential oils is that they do not increase antibiotic resistance compared to the long-term use of synthetic antibiotics. When bacteria are treated with essential oils, these oils degrade the cell membrane which is detrimental to the cytoplasmic membrane. Therefore, essential oils inhibit the growth of pathogens by targeting the membrane and cytoplasm and, in some cases, they totally change the structure of the bacteria. Essential oils have shown great potential in the biomedicine field and therefore their use in dentistry can be beneficial.

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CHAPTER THREE

MATERIALS AND METHODS

3.1 Introduction

Essential oils were selected based on previous research of antimicrobial activity of essential oils against oral pathogens (Takarada et al., 2004; Thosar et al., 2013; Mohapatra et al., 2020). The current research aimed at selecting the most effective essential oils against oral pathogens. The current study investigated the antimicrobial activity of five commercially accessible plant-derived essential oils, namely Lavandula latifolia(lavender), *Syzygium aromaticum* (clove bud), *Salvia officinalis* (sage), *Cinnamomum zeylanicum Blume* (cinnamon) and *Mentha piperita* (peppermint) against oral antibiotic-resistant bacteria namely *S. aureus, S. mutans* and *E. coli* pathogens, therefore the selected essential oils were investigated. Essential oils consist of different components that are responsible for inhibiting bacterial growth. Gas chromatography–mass spectrometry (GC-MS) was conducted to analyse the chemical composition of the five selected essential oils. The bioassay method, which is a commonly used agar dilution method and recommended by Kock et al. (2009), was used to measure the inhibition zone of bacteria on agar plates. The broth microdilution method as recommended by Desam et al. (2019) was used to assess the minimum inhibitory concentrations (MICs) of different essential oils against oral bacteria. Afterwards, scanning electron microscopy (SEM) was utilised to evaluate the structural changes of bacterial cells due to the application of the essential oils.

Ti6Al4V experimental dental implants were manufactured and the various essential oils' antimicrobial activities against oral bacteria were evaluated on the surfaces of the experimental dental implants using microdilution assay and scanning electron microscopy imaging to observe the structural changes caused by the essential oils to the cells of the bacteria.

3.2 Materials

3.2.1 Strains used

Microorganisms were obtained from Laboratory Specialities (Pty) Ltd, South Africa. The tested strains were *Staphylococcus aureus* (ATCC 25923), *Streptococcus mutans* (ATCC 25175) and *Escherichia coli* (ATCC 25922).

3.2.2 Antibacterial products used

Essential oils derived from *Lavandula latifolia* (lavender oil), *Syzygium aromaticum* (clove bud), *Salvia officinalis* (sage), *Cinnamomum zeylanicum Blume* (cinnamon) and *Mentha piperita* (peppermint) were purchased from local suppliers.

3.2.3 The powder used for manufacturing the experimental dental implant

Grade 23, Ti6Al4V extra low interstitial (ELI) alloy, which has low levels of oxygen, nitrogen, carbon and iron elements, was used for manufacturing the experimental dental samples. The low level of the interstitial elements enhances the ductility and increases the fracture toughness of the Ti6Al4V alloy (Gepreel and Niinomi 2013). Table 3.1 presents the chemical composition of the Ti6Al4V (ELI) alloy used for the manufacturing of the specimen. The morphology and particle size distribution of the Ti6Al4V (ELI) powder are presented in Figure 3.1 and Figure 3.2 respectively.

Table 3.1: **Chemical composition of Ti6Al4V (ELI) powder (in weight %)**

Ti		**Al**	**V**	**O**	**N**	**H**	**Fe**	**C**
	ASTM standard for Ti6Al4V (ELI) alloy grade 23							
88.1 - 91		5.5-6.5	3.5-4.5	0.13	0.030	0.0125	0.25	0.080
	Employed powder							
89.263		6.31	4.09	0.12	0.009	0.003	0.20	0.005

The powder used was spherical argon atomised powder (Figure 3. 1) procured by TLS Technik, Germany.

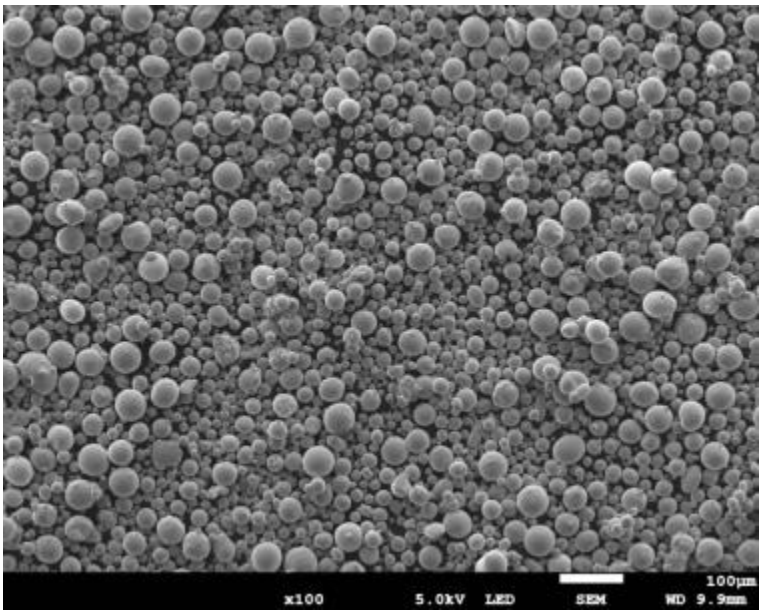

Figure 3.1: SEM micrograph of the Ti6Al4V powder

The 10th, 50th and 90th percentiles of equivalent diameter (weighted by volume) of the Ti6Al4V (ELI) powder were d_{10}=12.64 mm, d_{50}=22.93 mm and d_{90}=37.03 mm respectively (Figure 3.2).

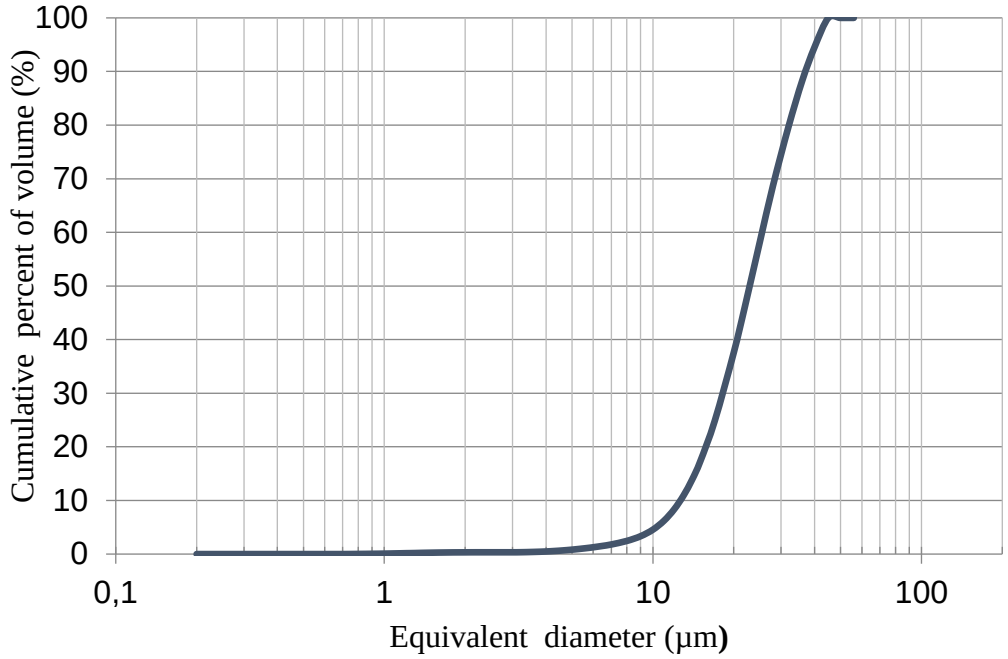

Figure 3.2: Particle size distribution of the Ti6Al4V ELI

3.3 Methods

3.3.1 Essential oil characterisation

Essential oils extracted from *Lavendula officinalis*, *Eugenia caryophyllids*, *Salvia officinalis, Cinnamomum zeylanicum Blume* and *Mentha piperita* were purchased from local suppliers. These essential oils were characterised according to the method proposed by Kirbaslar et al. (2009). Briefly, the oils were dissolved in hexane (10% hexane) and injected into a Finnigan focus gas chromatograph (GC) at a split ratio of 50:1. The injector temperature was set at 230°C. The GC was equipped with an AB-1MS (30m x 0.25µm) capillary column. Helium was used as carrier gas at a constant flow of 1mL min^{-1}. The temperature programme was set at 240°C for four minutes and then raised at 5˚C min^{-1} to 200˚C and then held at 200˚C for 1 minute and then raised at 220˚C where it was held for 10 min. Mass analysis of the oils was done using a Finnigan Focus DSQ mass spectrometer. The ion source was at 250˚C with an ionisation voltage of 70eV and mass scan range of 50-650 amu. Individual GC peaks and mass spectra were

identified by searching commercial libraries. This was followed by expert matching of MS data.

3.3.2 Bioassay preparation

A quantitative microbial bioassay technique was used to assess the antimicrobial activity of essential oils on bacteria growth. To make inocula, cultures were cultivated overnight at 37°C. Bacterial density was then adjusted to around 108 colony forming units (CFU) per ml for bioassay preparation. Bacterial strains used in bioassay were regrown on petri dishes and incubated for 24 hours at 37°C. Next, each bacterium was immersed in sterilised distilled water and 0.2 ml was spread out on PCA (0.5% m.v-1 agar). This created a homogenous lawn across the surface of the agar (Kock et al., 2009). Next, a hole (0.5 cm in diameter and depth) was created at the centre of each petri dish and 46 µl of the concentration of essential oils was added with ethanol. Ethanol (96%) was added to the wells alone as a control. All plates were incubated at 37°C for 24 hours until various textured growth zones were visible and then their inhibition zone diameters (mm) were measured. To avoid evaporation of the essential oils from the plates, the tested oils could diffuse in the agar before incubation.

3.3.3 Microdilution assay

In the conducted microdilution essay, the inoculate of the bacterial strains was prepared in Mueller-Hinton broth (MHB) for 24 hours, then suspensions of the 24-hour cultured bacteria in MHB broths were adjusted to 0.5 McFarland turbidity standard (approximately 1.5 x 108 cfu/mL). Each bacterial suspension was distributed into the 96-well sterile microtiter plate as demonstrated by Abidin et al. (2013) and Desam et al. (2019). Each essential oil was added to the first row of holes and serial dilutions were performed to reach final concentrations of 40, 20, 10, 5, 2.5 and 1. 25 µg/ml. The plate was enclosed with a sterile plate sealer and incubated at 37°C overnight. To show growth after 24 hours, the experiment was run twice in duplicate for each concentration. For incubation, 20 µl of

p-iodonitrotetrazolium violet (INT) was added to each well. The plate was then incubated at 37°C for 20 minutes. Growth was revealed by colour change varying from pink to violet.

3.3.4 Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) was used to evaluate the structural changes that occurred due to the substantial activity of *Cinnamomum zeylanicum Blume* and *Salvia officinalis* essential oils on the antibiotic-resistant *S. aureus, E. coli* and *S. mutans* bacteria in terms of shape and structure. Preparation of cells for examination using SEM was carried out as proposed by Ncango et al. (2010). Treated and untreated bacteria cells obtained from the microdilution assay method were principally fixed using 3% v/v of a sodium phosphate buffered glutaraldehyde solution at pH 7.0 and a similarly buffered solution (1% m/v) of osmium tetroxide for an hour. Afterwards, the samples were dehydrated in a graded series of ethanol solution (30%, 50%, 70%, 90% and 100% for 20 min per solution and the 100% dehydration was done twice for an hour). Next, the dehydrated samples with ethanol were critically dried and put on filter paper and coated with uranium to create electric conductivity. The coated samples were then evaluated using SEM. Images were taken to investigate the structural changes caused by the essential oils to the bacteria cells.

3.3.5 Manufacturing of the experimental Ti6Al4V dental samples

The Electro Optical System (EOS M280), a Direct Metal Laser Sintering (DMLS) machine and Additive Manufacturing (AM) technology, which belongs to the family of Laser Powder Bed Fusion (LPBF) processes, was used to manufacture the experimental implant samples. The DMLS machine chiefly encompasses a process chamber with a recoating system, computer control elevating system, platform heating module, an optical system equipped with 400-watt fibre laser, a process gas management system and a process computer with control software (Chua et al., 2010). The DMLS manufacturing technology is a subset of additive manufacturing (AM), which is colloquially known as 3D printing (Tao and Leu, 2016).

The DMLS technology is considered a distractive technology which is the renaissance of the manufacturing industry in this field (Dzogbewu, 2017a; Dzogbewu, 2020a). It is a monolithic manufacturing technology that has the unique capability of building complex geometries (e.g., back tapers, intricate cooling channels, customised porous structures and special lattices or hollow structures) which were impossible to manufacture with conventional methods of manufacturing (Yadroitsev et al., 2009). The DMLS process uses a layer-by-layer manufacturing strategy to produce 3D objects from single tracks which are arranged according to the computer aided designed (CAD) model (Dzogbewu et al., 2017b, Dzogbewu, 2020b). The essential manufacturing process involves a laser beam scanning the surface of a thin powder layer deposited on a substrate. The substrate is lowered after each laser scanning process until the 3D object is manufactured according to the CAD model. The manufacturing process is basically an eco-design topology optimisation technology that allows very complex parts to be created additively monolithically (Dzogbewu et al., 2017) as opposed to conventional methods of subtractive fabrications. The technology provides the designing engineer unchallenged freedom of design and the ability to manufacture functional biomimetic implants with tailored intricate customised architecture (Chua et al., 2010). The superior manufacturing capability of the DMLS process as equated to the classical methods of manufacturing permits the manufacturing of biological objects with tailored mechanical properties for specific biomedical applications. The DMLS has ushered in the manufacturing of prostheses with human bone-like biomechanical properties which has tremendously improved the life of patients' implants (Gepreel and Niinomi, 2013).

The substrate and powder materials used for the manufacturing of the experimental dental samples were similar in chemical composition. Argon was used as the protective atmosphere and the oxygen level in the chamber was 0.07–0.1%. The samples were manufactured with optimum process parameters: a laser power of 170 W, scanning speed of 1.25 m/s, hatch distance of 80 µm and powder layer thickness of 30 µm with zigzag scanning strategy (Dzogbewu et al., 2016). The samples were stress relieved in Ar atmosphere at a temperature of 650°C for 3 hours and were cut from the base plates

using electrical discharge machining. The samples were cleaned in an ultrasonic bath to remove the loosely attached powder residues.

3.3.6 Microdilution assay on titanium implant materials

Due to the significant antimicrobial activity of cinnamon and sage essential oils, minimum inhibition concentrations were conducted on the experimental titanium implant samples. The inoculate of the bacterial strains were made in Mueller-Hinton Broth (MHB) for 24 hours, then suspensions of the 24-hour cultured bacteria in MHB broth were adjusted to 0.5 McFarland turbidity standard (approximately 1.5 x 10^8 cfu/mL). Each bacterial suspension was distributed into glass tubes containing implant material (Abidin et al., 2013). Essential oils of cinnamon and sage were added to the first row of glass tubes and serial dilutions were performed to achieve final concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 µg/ml. The glass tubes were then incubated in a shaker incubator at 37°C overnight. To show growth after 24 hours, the experiment was run twice in duplicate for each concentration. Thereafter, 20 µl of p-iodonitrotetrazolium violet (INT) was added to each glass tube. The glass tubes were then incubated at 37°C for 20 minutes. Growth was revealed by colour change varying from pink to violet**.**

3.3.7 Scanning electron microscopy (SEM) on the titanium implant materials

Scanning electron microscopy (SEM) was used to assess the structural changes that had occurred due to the antimicrobial activity of *Cinnamomum zeylanicum Blume* and *Salvia officinalis* essential oils on antibiotic-resistant *S. aureus, E. coli* and *S. mutans* on the surface of the titanium implant material. Preparation of cells for evaluation using SEM was carried out according to the protocols proposed by Ncango et al. (2010). Treated and untreated bacteria cells on the surface of the titanium implant material (using the microdilution assay method) were primary fixed using 3% v/v of a sodium phosphate buffered glutaraldehyde solution at pH 7.0 0 and a similarly buffered solution (1% m/v) of osmium tetroxide for an hour. Consequently, the titanium implant material was dehydrated in a graded series of ethanol solution (30%, 50%, 70%, 90% and 100% for

20 min for each solution and the 100% dehydration was done two times for an hour). Next, the dehydrated titanium implant material with ethanol was heated at 40°C and then sputter coated with uranium for 30 min to create electrical conductivity.

The coated implant was then examined using SEM. Images were taken to investigate the structural changes caused by essential oils on the bacteria cells.

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CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 The Chemical Composition of the Selected Essential Oils

It is important to consider the chemical components of essential oils as the mode of action of these oils mainly depends on their chemical components. Table 4.1 shows the chemical components of the selected essential oils that were analysed using gas chromatography-mass spectrometry (GC-MS). Tariq et al. (2019) note that essential oils comprising aldehydes or phenols such as linanaldehyde, citral carvacrol, eugenol or thymol as major compounds possess the highest antibacterial activity, followed by essential oils containing terpene alcohol. In the current study, the main components of the essential oils were found to be terpenes and phenols. Cinnamon and clove essential oils had higher composition of phenols such as eugenol with 56.608% chemical composition in cinnamon essential oil and 63.921% chemical composition in clove essential oil. Table 4.1 also shows that the chemical composition of different essential oils varied significantly. The other main components of cinnamon oil were trans-cinnamaldehyde (14.165%) and eugenyl acetate (5.706%). Sage essential oil also contained terpenes such as β-Myrcene (13.62%), β-Phellandrene (11.02%) and bornanone (camphor) (6.79%) as its major constituents. Similar chemical components were previously recorded by Tu et al. (2018). Farhat et al. (2016) also reported that sage essential oil extracted from *Salvia officinalis* consisted of camphor and 1.8-cineole as the major chemical constituents that are known to inhibit human bacterial pathogens such as *Staphylococcus aureus* and *Providencia stuarti*. *Lavandula officinalis* contained 3.1% of camphene as a major constituent. It is for this reason that the compound is known to disrupt the cell membrane of bacteria and inhibit biofilm formulation (Leong et al., 2021). The terpenes found in essential oils are known to play a major role in the antimicrobial activity of essential oils (see the terpenes of the essential oils in Table 4.1) (Tu et al., 2018). The compounds identified in the essential oils were confirmed by comparing the results with those of previous studies (Zhang et al., 2016; Mahdavi et al., 2018). However, their chemical compositions differed, which could be attributed to different growth

conditions of the plants, genetic factors, chemical forms, harvesting periods and plant nutritional status. *Mentha piperita* essential oils (Table 4.1) consisted of menthol (33.05%), menthone (24.02%), menthofuran (12.57%) and neo-menthol (12.57%) as major constituents. A study conducted by Desam et al. (2019) observed similar results.

Table 4.1: **Chemical components of essential oils**

Essential oil %					
Compounds	***Cinnamomum zeylanicum Blume***	***Lavendula officinalis***	***Mentha piperita***	***Salvia officinalis***	***Syzygium aromaticum***
1,6 Octadien-3-ol, 3.7 dimethyl	-	7.7	-	-	-
4-Hexan-1-ol, 5 methyl-2-(1-methylethanyl) acetate	-	8.6	-	-	-
5-caranol, trans- (+)-	-	7.9	-	-	-
δ-3-Carene	-	-	-	9.62	-
α-Pinene	1.315	-	-	6.09	-
β-caryophyllene (trans)	3.184	-	2.53	-	7.245
β-Myrcene	0.152	-	-	13.62	-
Bornanone (Camphor)	-	-	-	6.79	-
Camphene	0.498	3.1	-	7.17	-

D-Limonene & β-Phellandrene	-	-	-	11.02	-
Eugenol	56.608	-	-	-	63.921
Eugenyl acetate	5.706	-	-	-	12.238
Isocryophillene	-	8.4	-	-	-
iso-menthone	-	-	12.57	-	-
menthofuran	-	-	12.57	-	-
menthol	-	-	33.05	-	-
menthyl acetate	-	-	8.90	-	-
menthone	-		24.02		
neo-menthol	-	-	12.57		
trans-Cinnamaldehyde	14.165	-	-	-	-

Mentha piperita consisted of 36.02% menthol, 24.56% menthone, 8.95% menthyl acetate and 6.88% menthofuran. The variations in chemical composition of the essential oils might have been due to the difference in geographical conditions, climate and effect of sunlight. Most studies that had been conducted earlier found that *Mentha piperita* consisted of high menthol as was observed in this study (Desam et al., 2019; Dias et al., 2019). Menthol is known to have antimicrobial activity against various bacteria such as *Staphylococcus epidermis* and *E. coli* (Martínez-Pabón and Ortega-Cuadros, 2020).

4.2 Antimicrobial Activity of Essential Oils

Bioassay was performed to select the essential oils with the highest inhibition diameters of at least 40 mm and above. These essential oils were applied to the surface of a titanium dental implant to explore their ability to prevent bacteria growth. Initially, five essential oils had been selected but only two, namely *Cinnamomum zeylanicum Blume* and *Salvia officinalis,* were found to be the most effective against *S. aureus, S. mutans and E. coli*. Each essential oil reacted differently on each bacterial strain and therefore each essential oil was selected for its effectiveness against a particular bacterial strain. For instance, *Cinnamomum zeylanicum Blume* showed remarkable inhibition on the growth of *S. aureus* and *E. coli and* therefore it was further investigated on only these two strains*. Salvia officinalis,* on the other hand, showed the highest inhibition on the growth of *S. mutans*. These two selected essential oils were further tested to determine their minimum inhibition concentrations (MICs) and the structural changes they caused on the bacteria cells. The current study thus concludes that these two essential oils may be effectively used on the surface of titanium dental implants to prevent bacterial growth.

4.2.1 Initial key results

The results of the different inhibition zones are presented in Table 4.2 and Figure 4.1-Figure 4.5. Clearly, the essential oils manifested different effectivity, which may have been due to the mode of action of each essential oil against the bacterial species that were investigated. According to Goni et al. (2009), the enrichment of fresh products with *Cinnamomum zeylanicum,* commonly known as cinnamon oil, is effective in reducing colonies of specific pathogens, especially fungi. This finding was corroborated when this essential oil was applied to bacteria that affect dental implants.

Table 4.2: **Inhibition zone diameters of essential oils against oral pathogens**

Essential oils	Bacteria inhibition zone diameters (mm)		
	Escherichia coli	***Staphylococcus aureus***	***Streptococcus mutans***
Cinnamomum zeylanicum Blume	45	40	35
Lavendula officianalis	20	5	20
Mentha piperita	30	30	10
Salvia officinalis	20	35	40
Syzygium aromaticum	16	6	20

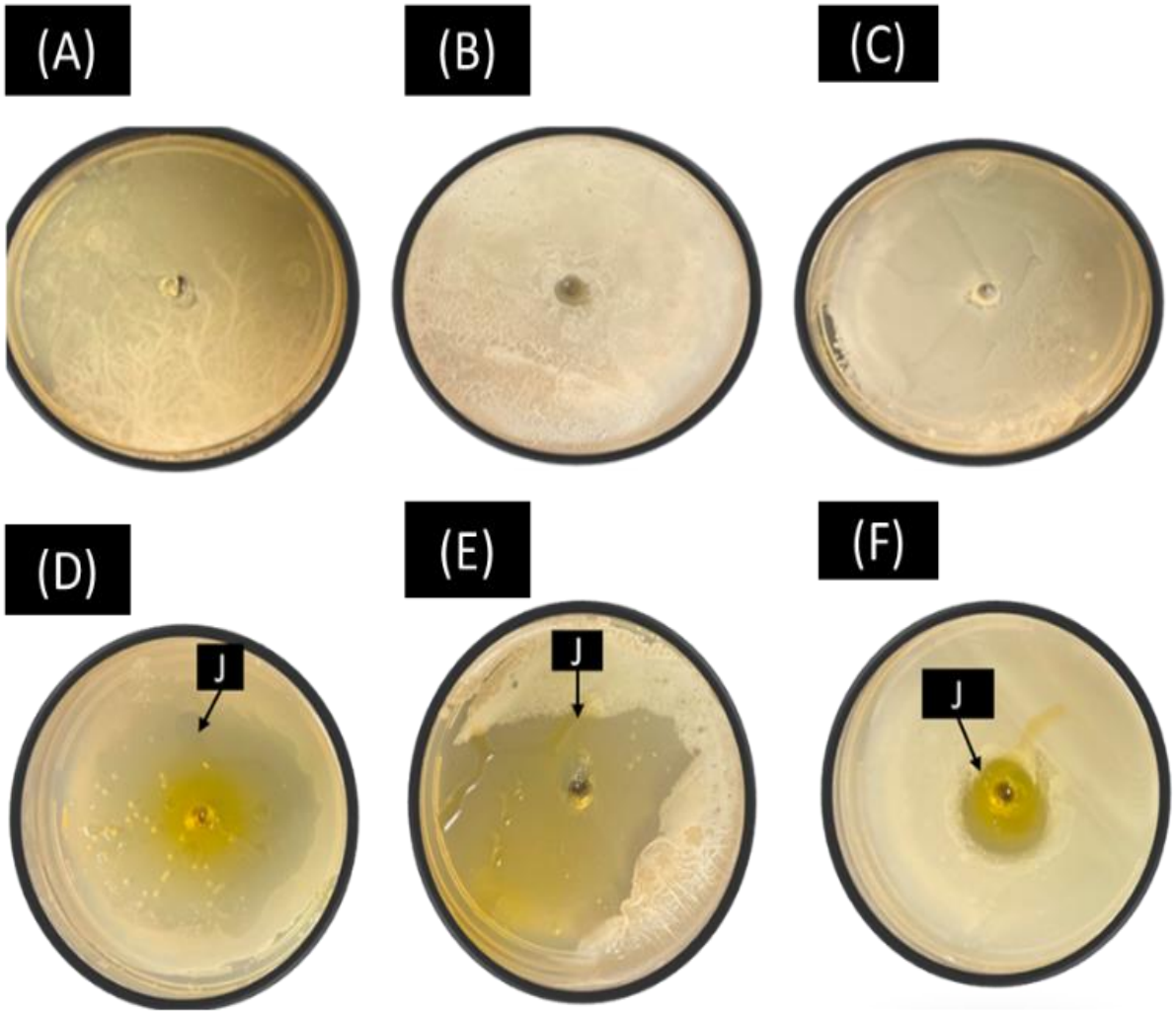

Figure 4.1: Bioassay of *Cinnamomum zeylanicum Blume* against *Escherichia coli, Staphylococcus aureus and Streptococcus mutan.* Controls: *Escherichia coli (A), Staphylococcus aureus (B) and Streptococcus mutans (C).* Treated: *Escherichia coli* (D), *Staphylococcus aureus (E) and Streptococcus mutans* (F). J-inhibition zone

Cinnamomum zeylanicum Blume essential oil showed significant antimicrobial activity against *S. aureus, E. coli and S. mutans* with an inhibition zone of 40 mm for *S. aureus*, 45 mm for *E. coli and* 35 mm for *S. mutans*. Similar results were obtained by Barajas et al. (2016), who found that *Cinnamomum zeylanicum Blume* was the most active against oral pathogens.

Conversely, *Lavandula latifolia* essential oil showed the least antimicrobial activity against *S aureus, E. coli and S. mutans* with inhibition zones of 5 mm on *S. aureus,* 20 mm *on E. coli and* 20 mm on *S. mutans* (Table 4.2 and Figure 4.2). As a result, this essential oil was not further tested or used on the surface of the titanium dental implant samples as it had not shown remarkable inhibition on the above-mentioned bacterial strains. However, earlier studies found that *Lavendula officianalis* (lavender) was accountable for damaging the cell wall and cytoplasmic membrane of bacteria such as *S. aureus* and *E. coli* and that it inhibited the growth of these bacteria (Chouhan et al., 2017). The literature also asserts that lavender essential oil has antimicrobial activity against *Enterococcus faecalis, Staphylococcus aureus, Candida albicans and Escherichia coli* (De Rapper et al., 2016; Chouhan et al., 2017). Unfortunately, as this essential oil did not achieve the desired result in the current study, its use was not further tested, as was explained before.

Mentha piperita (peppermint) essential oil was also initially analysed and it was found that it showed momentous antimicrobial activity against *S. aureus, E. coli* and *S. mutans* with inhibition zones of 30 mm on *S. aureus,* 30 mm on *E. coli and* 10 mm on *S. mutans* (Table 4.2 and Figure 4.3)*.*This essential oil is known to be remarkably powerful for fighting oral pathogens and slaying common bacteria such as *Acinetobacter baumenii, Escherichia coli, Streptococcus pyogenes, Enterococcus faecalis, Klebsiella pneumoniae and Pseudomonas aeruginosa* cause cavities and gum disease (Wamke et al., 2009; Shalal et al., 2017). Membrane permeability leakage of the cytoplasm and death by breaking the cell membrane of *E. coli* (Wamke et al., 2009; Dagli et al., 2015; Nam et al., 2018).

During bioassay preparation, *Salvia officinalis* (sage oil) also showed significant inhibitory effects (Table 4.2 and Figure 4.4.) with inhibition diameters of 40 mm for *Salvia officinalis* against *S. mutans*, 20 mm against *E. coli and* 35 mm against *S. aureus.*

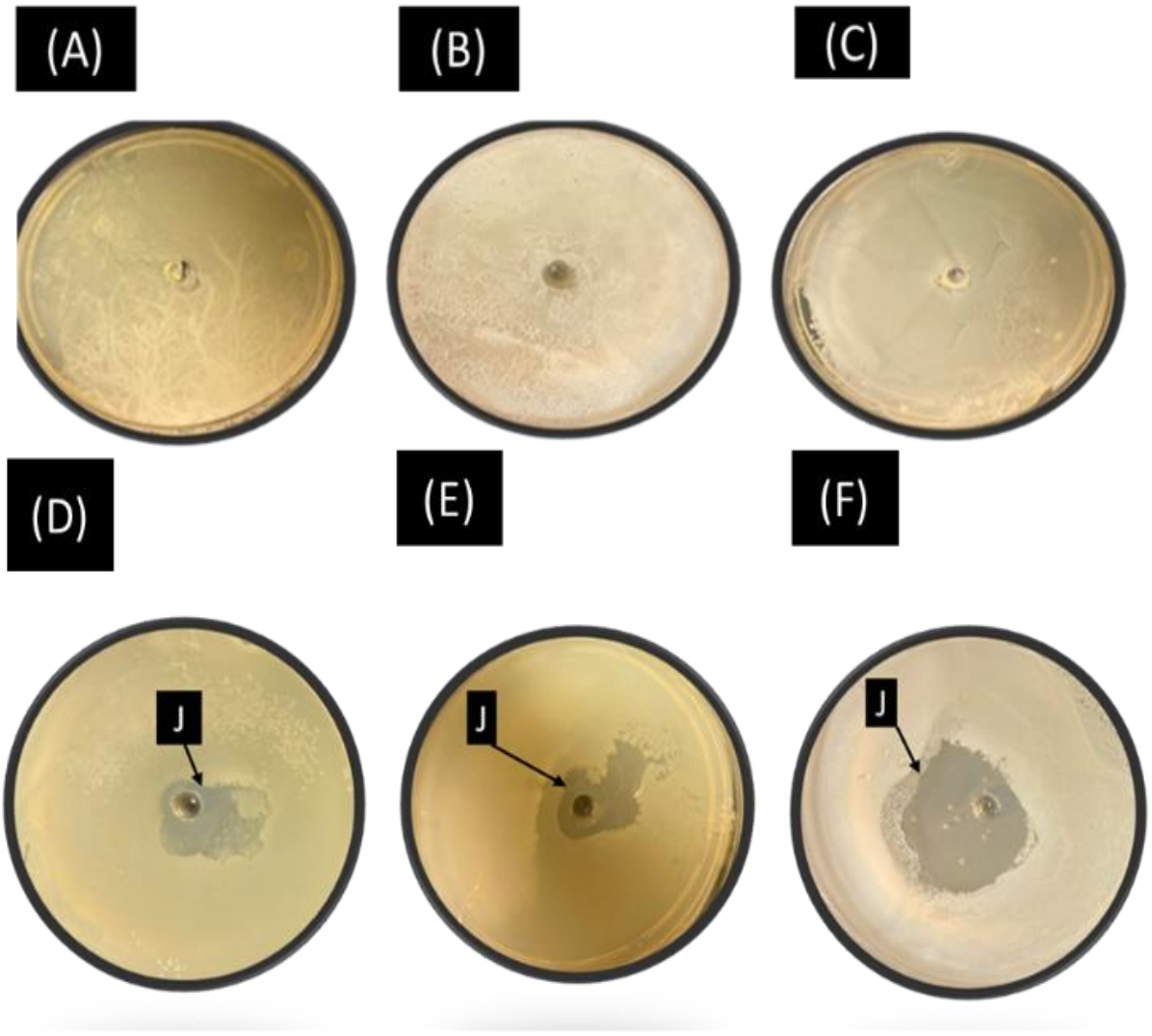

Figure 4.2: Bioassay of *Lavendula officianalis* against *Escherichia coli, Staphylococcus aureus and Streptococcus mutan*. *Controls: Escherichia coli* cell (A), *Staphylococcus aureus* cell (B) *and Streptococcus mutans* cell (C).
Treated: *Escherichia coli* cell (D), *Staphylococcus aureus* cell (E) *and Streptococcus mutans* cell (F). J-inhibition zone

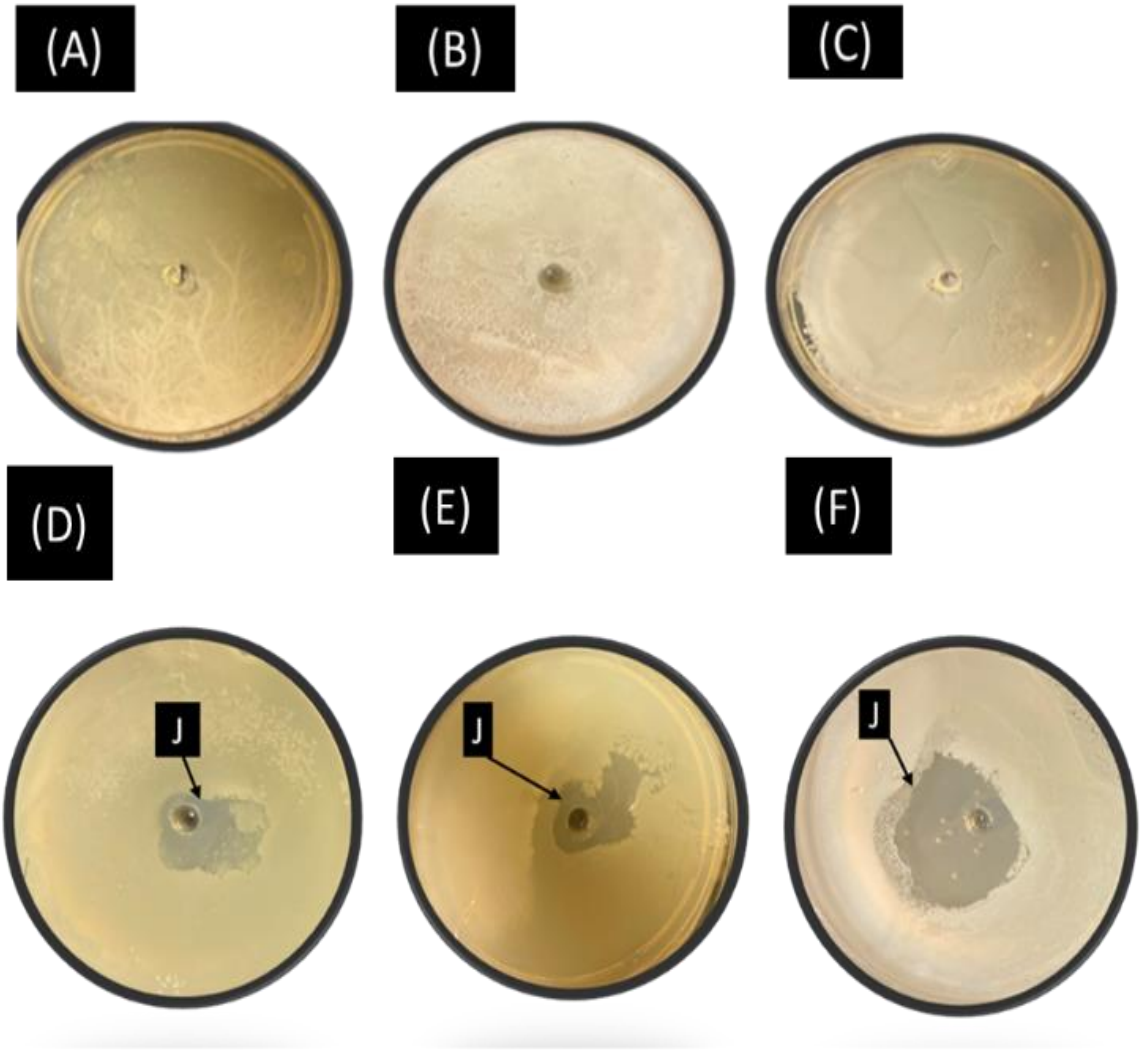

Figure 4.3: Bioassay of *Mentha piperita* against *Escherichia coli*, Staphylococcus *aureus* and *Streptococcus mutan.* Controls: *Escherichia coli cell A), Staphylococcus aureus cell (B) and Streptococcus mutans cell (C).* Treated: *Escherichia coli* cell (D), *Staphylococcus aureus* cell (E) *and Streptococcus mutans* cell (F). J-inhibition zone.

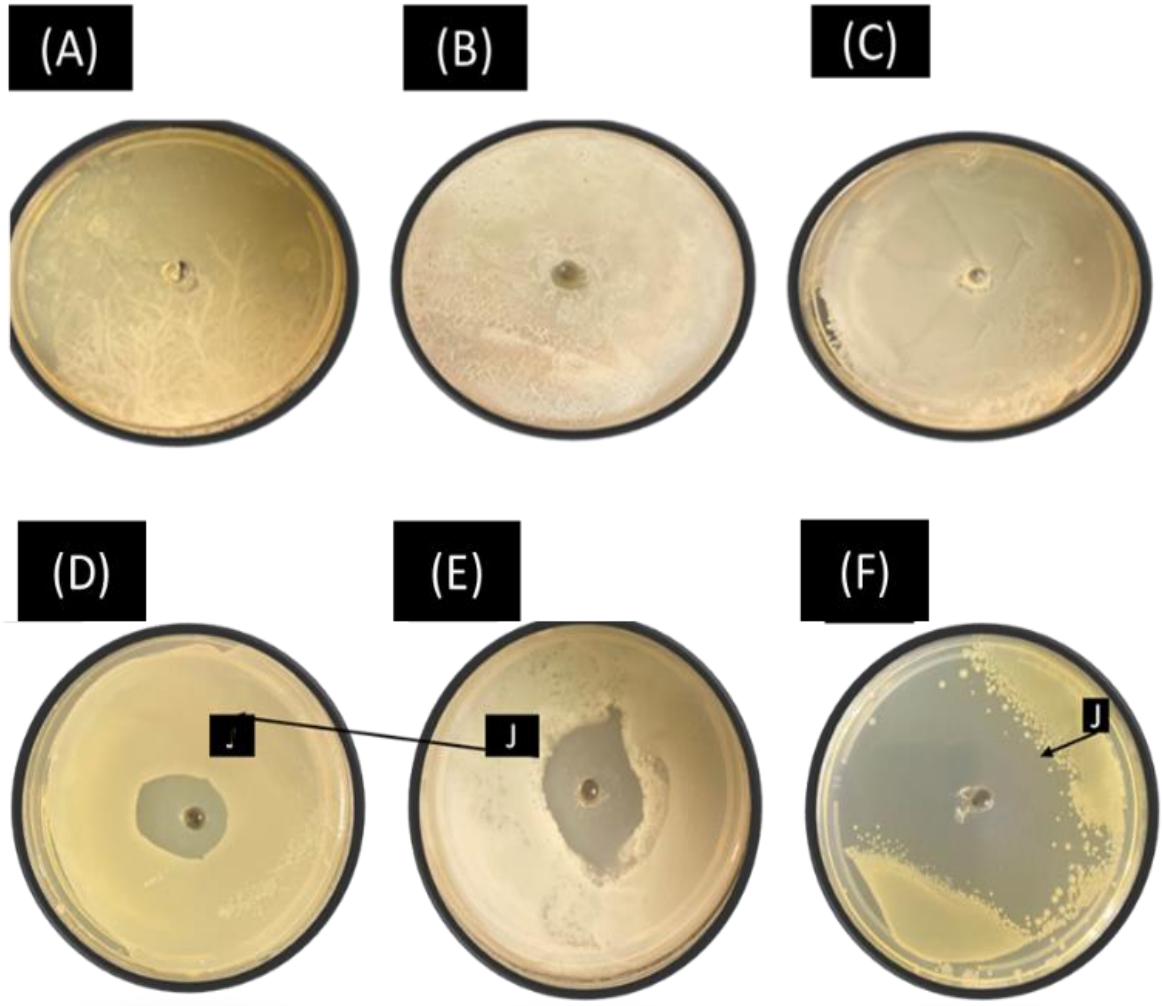

Figure 4.4: Bioassay of *Salvia officinalis* against *Escherichia coli, Staphylococcus aureus* and *Streptococcus mutan. Controls: Escherichia coli cell (A), Staphylococcus aureus cell (B) and Streptococcus mutans cell (C). Treated: Escherichia coli* cell (D), *Staphylococcus aureus* cell (E) and *Streptococcus mutans* cell (F). *J-inhibition zone*

The results demonstrated that *Salvia officinalis* essential oil had a very strong antibacterial activity. *Salvia officinalis* essential oil was mostly effective in inhibiting the growth of *S. mutans,* which is one of the primary pathogenic bacteria in dental caries (Popa et al., 2020). However, even though previous studies had found promising results regarding the antimicrobial activity of *Salvia officinalis*, the oil showed the lowest antimicrobial potential against *Candida*, which is a causative agent of several human oral disorders, when compared to other essential oils such as *H. officinalis* and *R. officinalis*

(Serra et al., 2018). In the current study, *Salvia officinalis* showed remarkable antimicrobial potential against *S. mutans.* In a similar study by Imane et al. (2020) in which the antimicrobial activity of sage oil was evaluated and it was found that the potential of sage as a therapeutic agent was outstanding as it showed the ability to control the development of opportunistic pathogens such *as S. aureus, S. epidermidis, S. mutans, C. albicans, C. tropicalis and glabrata* (Imane et al., 2020).

Apart from *Salvia officinalis* essential oil, *Syzygium aromaticum* (clove bud oil) was also selected in the current evaluation because it is known to be effective against oral bacteria and fungi such as *C. albicans, S. aureus and S. mutans* that are all associated with dental caries and periodontal disease (Warnke et al., 2009; Thosar et al., 2013; Nabavi et al., 2015). However, *Syzygium aromaticum* essential oil showed the least antimicrobial activity against *S. aureus, E. coli and S. mutans* with inhibition zones of only 6 mm for *S. aureus*, 16 mm for *E. coli and* 20 mm for *S. mutans* (Table 4.2 and Figure 4.5). For that reason, this essential oil (*Syzygium aromaticum*) was not used any further.

4.2.2 Further testing and results

The results that were obtained during bioassay preparation showed that the essential oils with the most significant antimicrobial activity could be further used for coating the titanium dental implants to test their ability to inhibit bacteria growth on this surface. In this phase of the study the oils with the least antimicrobial activity were eliminated. It is noteworthy that each essential oil reacted differently on each bacterial strain, which was attributed to the chemical components of each essential oil.

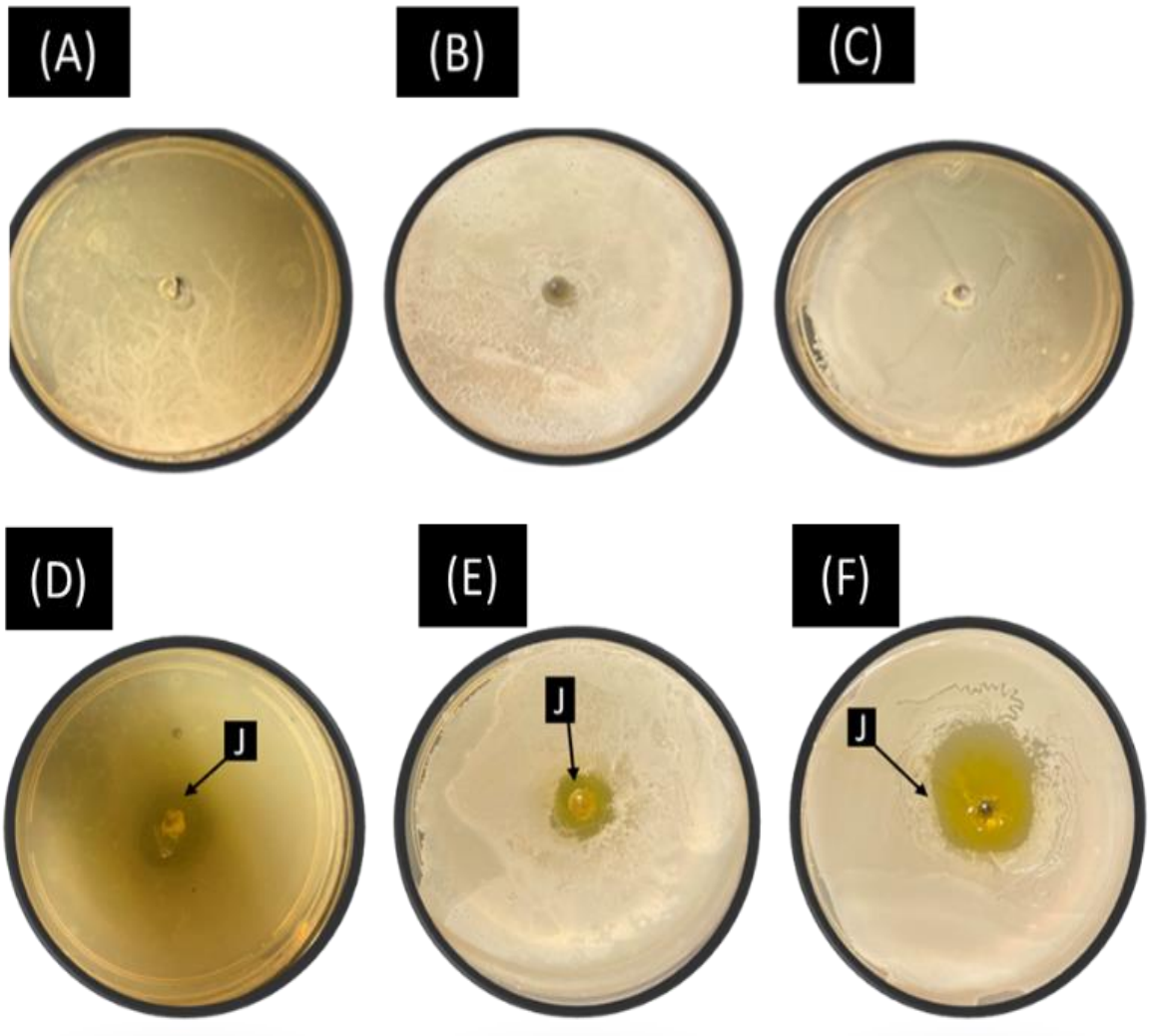

Figure 4.5: Bioassay of *Syzygium aromaticum* against *Escherichia coli, Staphylococcus aureus* and *Streptococcus mutan.* Controls: *Escherichia coli cell (A), Staphylococcus aureus cell (B) and Streptococcus mutans cell (C).* Treated: *Escherichia coli* cell (D), *Staphylococcus aureus* cell (E) and *Streptococcus mutans* cell (F). J-inhibition zone *controls:*

As was stated earlier, the results showed that, of the five essential oils that were tested, *Cinnamomum zeylanicum Blume* exhibited the most significant inhibitory effect against *S. aureus* and *E. coli,* whereas *Salvia officinalis* showed a significant inhibitory effect against *S. mutans.* As a result, these two essential oils were selected to be used on the surface of the titanium dental implants to prevent bacteria growth of *E. coli, S. aureus* and *S.*

mutans. However, prior to their use on the surface of the titanium dental implants, their minimum inhibition concentrations on bacteria were investigated using microdilution essay. *Cinnamomum zeylanicum Blume* essential oil had MICs of 10 µg/ml against *S. aureus* and 20 µg/ml against *E. coli* (Table 4.3 and Table 4.4). *Salvia officinalis* essential oil showed an MIC of 5 µg/ml against *S. mutans* (Table 4.5). The results thus suggested that *Salvia officinalis* essential oil had very strong antibacterial activity. According to Tardugno et al. (2018), sage essential oil is highly effective in inhibiting the growth of *S. mutans,* which is one of the primary pathogenic bacteria in dental caries. A study by Nikolić et al. (2016) showed that *Salvia officinalis* had the lowest antimicrobial potential against *Candida,* which is regarded as a causative agent for several human oral disorders. In fact, in the latter study other essential oils such as *Hyssopus officinalis* and *Rosmarinus officinalis* delivered better results. However, in the current study *Salvia officinalis* showed the highest antimicrobial potential against *S. mutans.* De Oliveira et al. (2019) conducted a similar study on the antimicrobial activity of sage oil and concluded that the potential of sage as a therapeutic agent was remarkable as it controlled the development of opportunistic pathogens such *as S. aureus, S. epidermidis, S. mutans, C. albicans, C. tropicalis and glabrata*.

Table 4.3: Minimum inhibitory concentration of *Cinnamomum zeylanicum Blume* against *E. coli* isolate

***Cinnamomum zeylanicum Blume* and control (µg/ml)**	***≥40***	***≥20***	***≥10***	***≥ 5***	***≥2.5***	***≥1.25***
Cinnamomum zeylanicum Blume	-	-	+	+	+	++
control	+++	+++	+++	+++	+++	+++

Data that are reported as '+' indicate growth of bacteria (not sensitive to *Cinnamomum zeylanicum Blume* oil) while '–' indicates inhibition of growth of bacteria (sensitive to *Cinnamomum zeylanicum Blume* oil).

Table 4.4: Minimum inhibitory concentration of *Cinnamomum zeylanicum Blume* against *S. aureus* isolate

***Cinnamomum zeylanicum Blume* and control (µg/ml)**	**≥40**	**≥20**	**≥10**	**≥ 5**	**≥2.5**	**≥1.25**
Cinnamomum zeylanicum Blume	-	-	-	+	+	++
control	+++	+++	+++	+++	+++	+++

Data that are reported as '+' indicate growth of bacteria (not sensitive to *Cinnamomum zeylanicum Blume* oil) while '–' indicates inhibition of growth of bacteria (sensitive to *Cinnamomum zeylanicum Blume* oil).

Table 4.5: Minimum inhibitory concentration of *Salvia officinalis* against *S. mutans* isolate

***Salvia officinalis* and control (µg/ml)**	**≥40**	**≥20**	**≥10**	**≥ 5**	**≥2.5**	**≥1.25**
Salvia officinalis	-	-	-	-	+	++
control	+++	+++	+++	+++	+++	+++

Data that are reported as '+' indicate growth of bacteria (not sensitive to *Salvia officinalis* oil), while '–' indicates inhibition of growth of bacteria (sensitive to *Salvia officinalis* oil).

4.3 Morphological changes of bacteria cells in the presence of essential oils.

Antibiotic resistance is a main public health challenge because microorganisms can survive and replicate even in the presence of antimicrobial agents such as antibiotics (WHO, 2014). For instance, bacteria use efflux pumps to push out the antimicrobial agent,

thus preventing it from entering the cell (WHO, 2016). Therefore, essential oils are deemed very effective antimicrobial agents because they can penetrate through the bacterial cell and destroy the bacterial cell wall while damaging the cytoplasmic membrane and prevent cytoplasm coagulation (Sridhar et al., 2015; Zhang et al., 2016). Alteration in the structure of *Staphylococcus aureus, Streptococcus mutans* and *Escherichia coli* was observed after treated with minimum inhibition concentration of *Cinnamomum zeylanicum Blume* and *Salvia officinalis* essential oils. These structural changes caused by the essential oils were detected under the scanning electron microscope (SEM) and the results are shown in Figure 4.6 to Figure 4.8. Significant alterations were observed on and in the bacterial cells with regards to their shape and size when treated with the selected essential oils. Tariq et al. (2019) argue that essential oils can easily gain entrance to a cell through the bacterial cell membrane and thus damage the entire cell. In the current study, the SEM results showed that *Cinnamomum zeylanicum Blume* and *Salvia officinalis* essential oils increased bacterial cell membrane permeability of *Staphylococcus aureus, Streptococcus mutans and Escherichia coli,* which resulted in leakage of cellular content. More particularly, *Cinnamomum zeylanicum Blume* and *Salvia officinalis* essential oils were able to penetrate through the bacterial cell wall of *Staphylococcus aureus, Streptococcus mutans* and *Escherichia coli* causing damage to the entire bacterial cell.

Figure 4.6 clearly indicates that the structure of the *S. mutans* bacterial cell was damaged. When treated with 5 µg/ml of *Salvia officinalis* essential oil, the cell damage that was caused resulted in the loss of cellular content, cell wall division and roughage. The results showed that some cells had collapsed and lysed, which probably caused leakage of the intracellular contents. These observations suggest that the mechanism of antibacterial activity of *Salvia officinalis* essential oil may be associated with the disturbance of the membrane structure or cell wall of the bacterium cell upon contact.

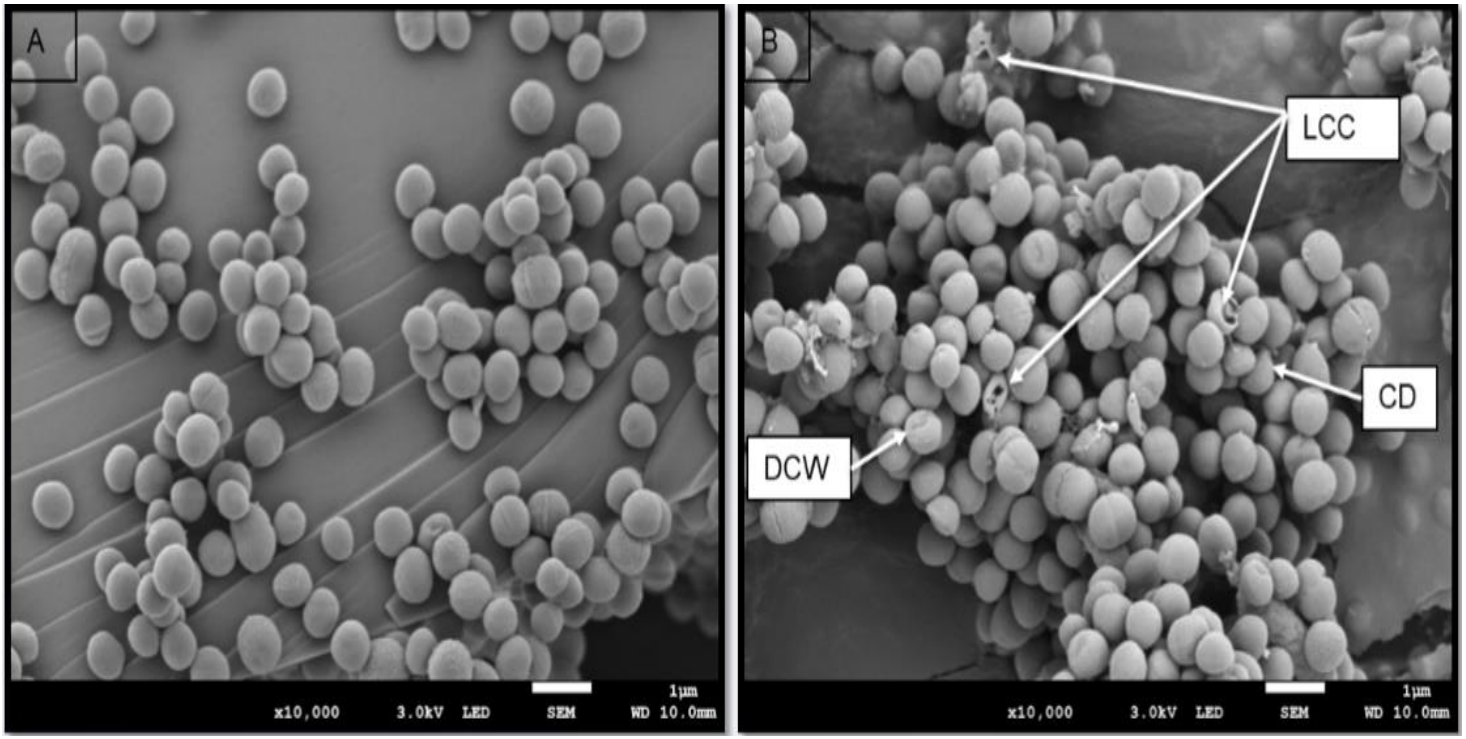

Figure 4.6: Scanning electron microscope *image* A of *S. mutans* cells control. Image B shows *S. mutans* cells treated with 5 µg/ml of *Salvia officinalis* oil, showing loss of cell content (LCC), cell division (CD) and damaged cell wall (DCW).

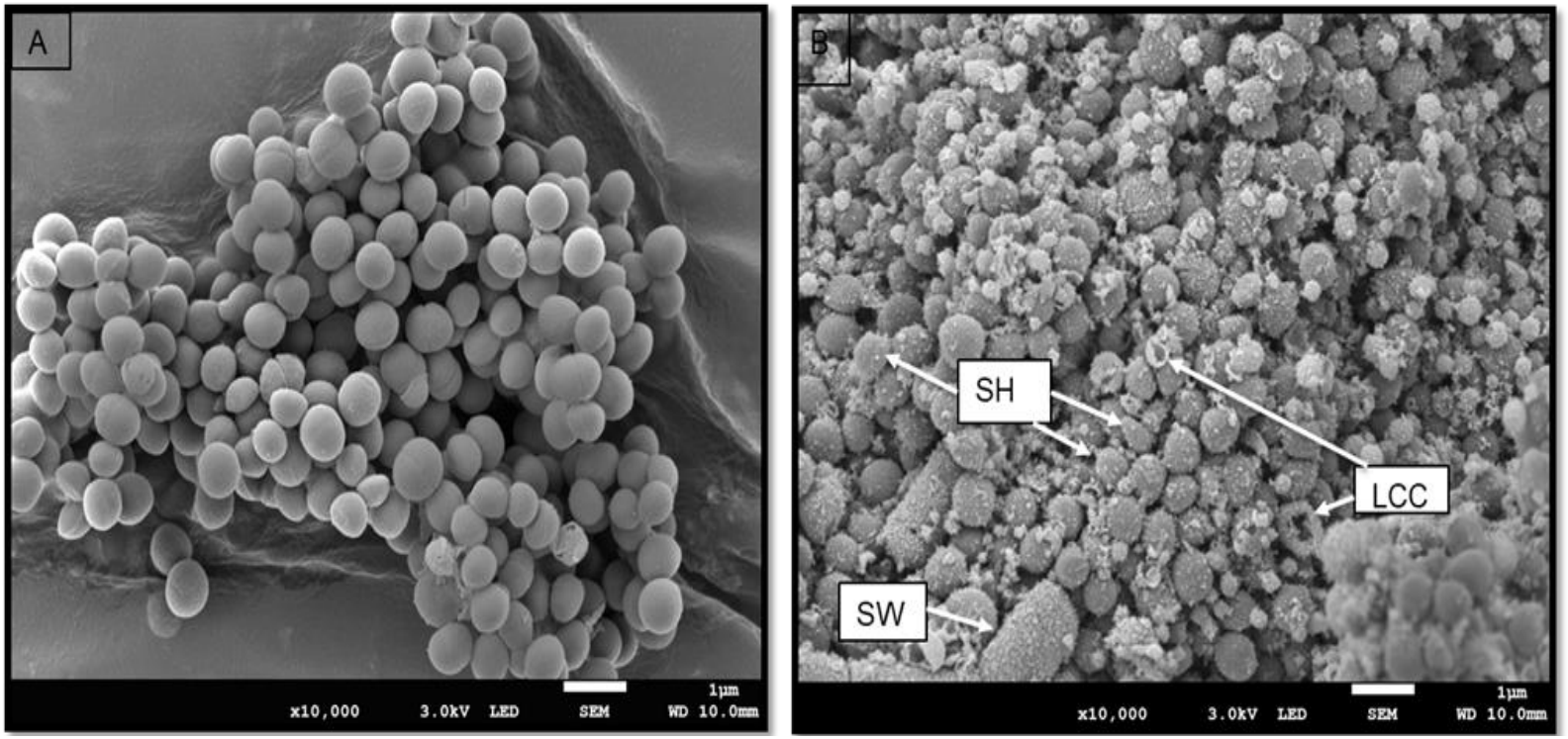

Figure 4.7: Scanning electron microscope *image A* of *S. aureus* cells control. Image B shows *S. aureus* cells treated with 10 µg/ml. of *Cinnamomum zeylanicum Blume*, showing swollen bacteria cells (SW), shrinkage of bacteria cells (SH) and Loss of cellular content (LCC).

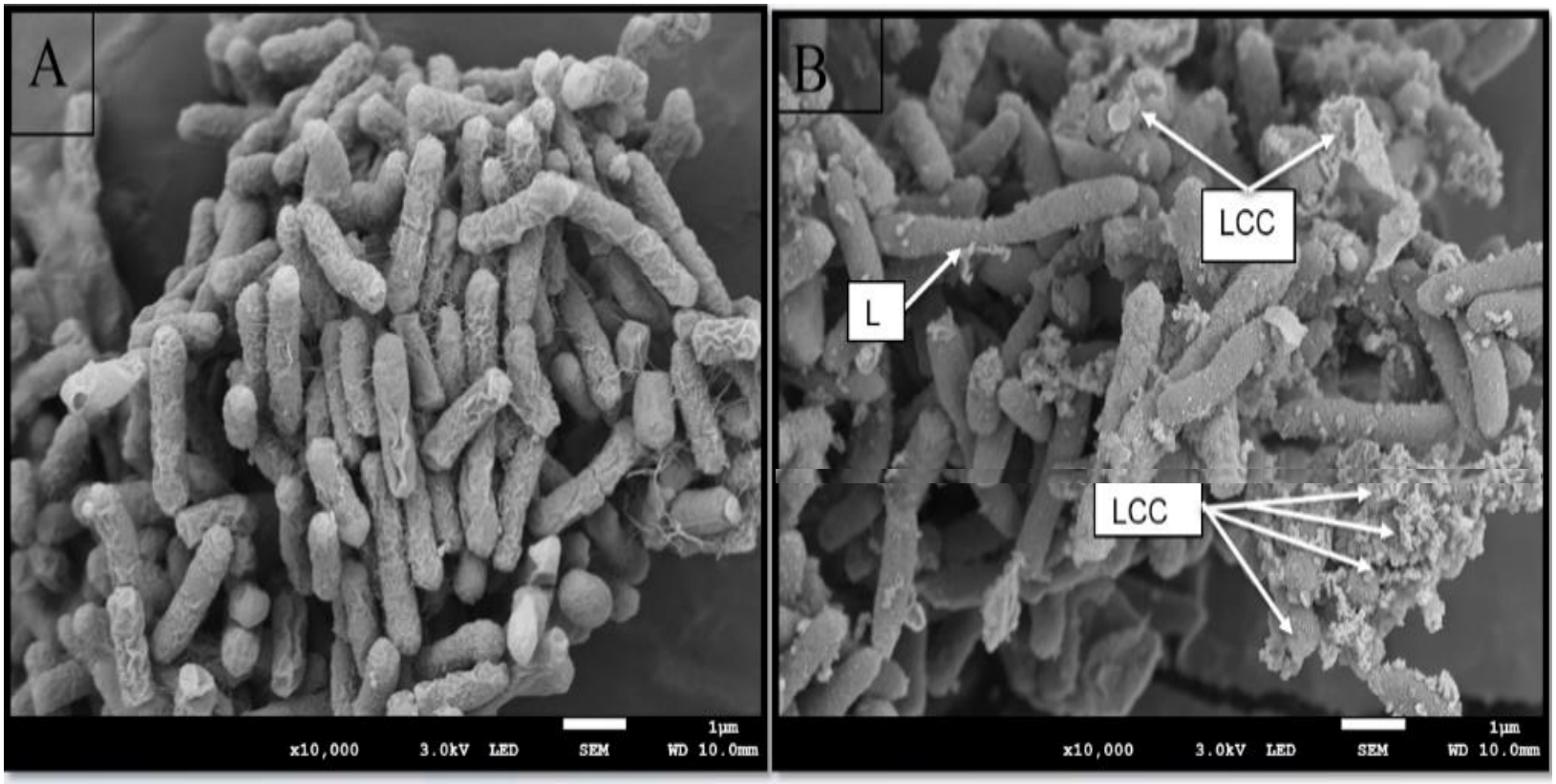

Figure 4.8: Scanning electron microscope image A of *E. coli* cells control. Image B shows *E. coli* cells treated with 20 µg/ml of *Cinnamomum zeylanicum Blume* oil, showing loss of cell content (LCC) and elongated cell (L).

Radical cell structural alterations upon treatment with *Cinnamomum zeylanicum Blume* against *S. aureus* with 10 µg/ml were observed when compared to the control. *Cinnamomum zeylanicum Blume* essential oil targeted the synthesis of cell walls and cell membranes as observed in Figure 4.7, where *S. aureus* cell walls were damaged leading to loss of cellular content. It is possible that the inhibition of or damage to the cell walls may have caused the *S. aureus* bacterial cells to lose control over their structure, thus altering their shapes and sizes. Moreover, further disruption in homeostasis led to shrinkage and eventually the death of the affected cells. *Cinnamomum zeylanicum Blume* essential oil also caused roughage and a high loss of cellular content. Husain et al. (2015) believe that the chemical components of cinnamon oil, such as cynammyldehyde and eugenol, account for the inhibited growth of *S. aureus*.

Figure 4.8 also shows extreme structural changes to the *E. coli* cells when treated with 20 µg/ml of *Cinnamomum zeylanicum Blume* compared to the control. In comparison with normal bacterial cells, the treated cells indicated pleomorphic, abnormal size and some had ruptured. The bacterial cells also presented irregular shape and elongated after treatment with *Cinnamomum zeylanicum Blume* as shown in Figure 4.8. Similar results were noted in a study that was conducted by Zhang et al. (2016), where *Cinnamomum zeylanicum Blume* oil destroyed the bacteria cell walls leading to leakage of cellular content.

4.4 Antimicrobial Activity of Essential Oils on Ti6Al4V Dental Implants

Bioassay preparation was performed to select the most effective essential oils against *E. coli, S. aureus and S. mutans.* These oils were then applied to the surface of the experimental titanium dental implants (*Cinnamomum zeylanicum Blume* was used to inhibit the growth of *E. coli* and *S. aureus* and *Salvia officinalis* was used to inhibit the growth of *S. mutans*). These essential oils had reportedly been used to treat bacteria that colonised the surface of titanium implant materials and that had caused implant failure at an early stage of insertion. Coating implant materials with antibiotics, antiseptics or any antimicrobial agent can inhibit bacterial growth on the surfaces of such implants (Khatoon

et al., 2018; Woo et al., 2020), therefore the current study investigated the use of essential oils with the aim of inhibiting bacteria growth on the surface of titanium dental implants.

The antimicrobial activity of *Cinnamomum zeylanicum Blume* and *Salvia officinalis* essential oils on the surface of titanium dental implant material was qualitatively evaluated by obtaining minimum inhibition concentrations (MICs) of these oils to determine their effect against *S. aureus, S. mutans and E. coli*. The microdilution method was used. The titanium implant material was soaked in solutions containing these essential oils respectively to investigate the minimum inhibition concentration (MICs) of *Cinnamomum zeylanicum Blume* and *Salvia officinalis* against *S. aureus, S. mutans and E. coli*. A *Cinnamomum zeylanicum Blume* essential oil solution with a MIC of 25 µg/ml was prepared for treating *S. aureus,* while a *solution* of 50 µg/ml of this oil was prepared for treating *E. coli.* Also, a solution of 12.5 µg/ml was prepared to treat *S. mutans* cells using *Salvia officinalis* essential oil (Table 4.6 -Table 4.8). The essential oils were used at their minimum concentrations to investigate the structural changes they caused on and inside the bacteria cells. Scanning electron microscopy (SEM) was employed to observe at what morphological level the two essential oils affected *S. aureus, S. mutans and E. coli* isolates.

4.5 Structural Changes Caused by the Selected Essential oils to Bacteria Cells Plated on Ti6Al4V Experimental Implants

The oral pathogens were treated with the essential oils at their respective MIC values. The images were taken after the implants were incubated in a shaker incubator at 37°C overnight (24 hours). Therefore, the results are presented in Figure 4.9 to Figure 4.11. The images reveal that the essential oils caused changes to the bacterial cells with regards to their shape, size and arrangement. These observations suggest that the mechanism of antibacterial activity of both oils contributed to the disruption of the bacteria membrane structure or cell walls upon contact.

The *S. aureus, S. mutans* and *E. coli* bacteria showed drastic structural alterations upon treatment with *Cinnamomum zeylanicum Blume* and *Salvia officinalis*. It is thus possible that the visible destruction of the cell walls of the *S. aureus, S. mutans* and *E. coli* bacteria may have caused the cells to lose control over their shape and size and that it may also have caused the destructive loss of cellular content.

Table 4.6: Minimum inhibitory concentration of *Cinnamomum zeylanicum Blume* against *E. coli*

***Cinnamomum zeylanicum Blume* and control (µg/ml)**	**≥100**	**≥50**	**≥25**	**≥ 12.5**	**≥6.25**	**≥3.125**
Cinnamomum zeylanicum Blume	-	-	+	+	++	++
control	+++	+++	+++	+++	+++	+++

Data that are reported as '+' indicate growth of bacteria (not sensitive to *Cinnamomum zeylanicum Blume* oil), while '–' indicates inhibition of growth of bacteria (sensitive to *Cinnamomum zeylanicum Blume* oil).

Table 4.7: Minimum inhibitory concentration of *Cinnamomum zeylanicum Blume* against *S. aureus*

***Cinnamomum zeylanicum Blume* and control (µg/ml)**	**≥100**	**≥50**	**≥25**	**≥ 12.5**	**≥6.25**	**≥3.125**
Cinnamomum zeylanicum Blume	-	-	-	+	+	++
control	+++	+++	+++	+++	+++	+++

Data that are reported as '+' indicate growth of bacteria (not sensitive to *Cinnamomum zeylanicum Blume* oil), while '–' indicates inhibition of growth of bacteria (sensitive to *Cinnamomum zeylanicum Blume* oil).

Table 4.8: Minimum inhibitory concentration of *Salvia officinalis* against *S. mutans*

***Salvia officinalis* and control (µg/ml)**	**≥100**	**≥50**	**≥25**	**≥ 12.5**	**≥6.25**	**≥3.125**
Salvia officinalis	-	-	-	-	+	++
control	+++	+++	+++	+++	+++	+++

Data that are reported as '+' indicate growth of bacteria (not sensitive to *Salvia officinalis* oil), while '–' indicates inhibition of growth of bacteria (sensitive to *Salvia officinalis* oil).

S. mutans was treated with 12.5 µg/ml of *Salvia officinalis* oil at its respective MIC values (Table 4.8). The SEM results are shown in Figure 4.9. There were striking alterations to the bacterial cells with regards to their shape and size when treated with the essential oils. Tariq et al. (2019) believe that essential oils can easily penetrate bacterial cell membranes and similar results were obtained in the current study. Loss of cell contents, cell division, damaged cell walls, elongated cells and roughage of the bacteria cells plated onto the surface of titanium implants were observed. These observations suggest that the mechanism of antibacterial activity of *Salvia officinalis* essential oil with its major compounds β-Myrcene (13.62%), δ-3-Carene (9.62%) and 2-Bornanone (camphor) 6.79% (Table 4.1) could be related to the disturbance of membrane structure or cell wall inhibition of the bacteria upon exposure. The literature asserts that *Salvia officinalis* consists of α-thujone, camphor and 1.8-cineole as its major chemical constituents and is known to inhibit human bacterial pathogens such as *S. aureus* and *Providencia stuarti.* Similar results were observed in the current study as this essential oil consists of camphor and inhibited bacteria growth of *S. mutans* (Farhat al., 2016). *Salvia officinalis* thus exerts a variety of therapeutic activities as it has antibacterial, antiviral, antifungal and antioxidant effects. The findings of the current study thus suggest that *Salvia officinalis* could also be beneficial in the dentistry field, as Beheshti-Rouy et al. (2015) propose.

Cinnamomum zeylanicum Blume essential oil consists of a high composition of phenols such as eugenol with a 56.608% chemical composition (Table 4.1). As was previously

mentioned, essential oils containing phenols such eugenol as a major compound exert the highest antibacterial activity, followed by essential oils containing terpene alcohol (Tariq et al., 2019). The current study demonstrated that cinnamon essential oil containing 56.608% eugenol had significant antimicrobial activity against oral bacteria such as *Staphylococcus aureus* and *Escherichia coli.* Cui et al. (2016) agree that eugenol and eugenyl acetates are the major constituents of cinnamon oil and are responsible for disrupting the membranes of bacterial cells, leading to cell death. Therefore, the current study demonstrated that, of the five selected essential oils, *Cinnamomum zeylanicum Blume officinalis* had the most significant (Figure 4.1) with inhibition diameters of 45 mm against *E. coli* and 40 mm against *S. aureus*.

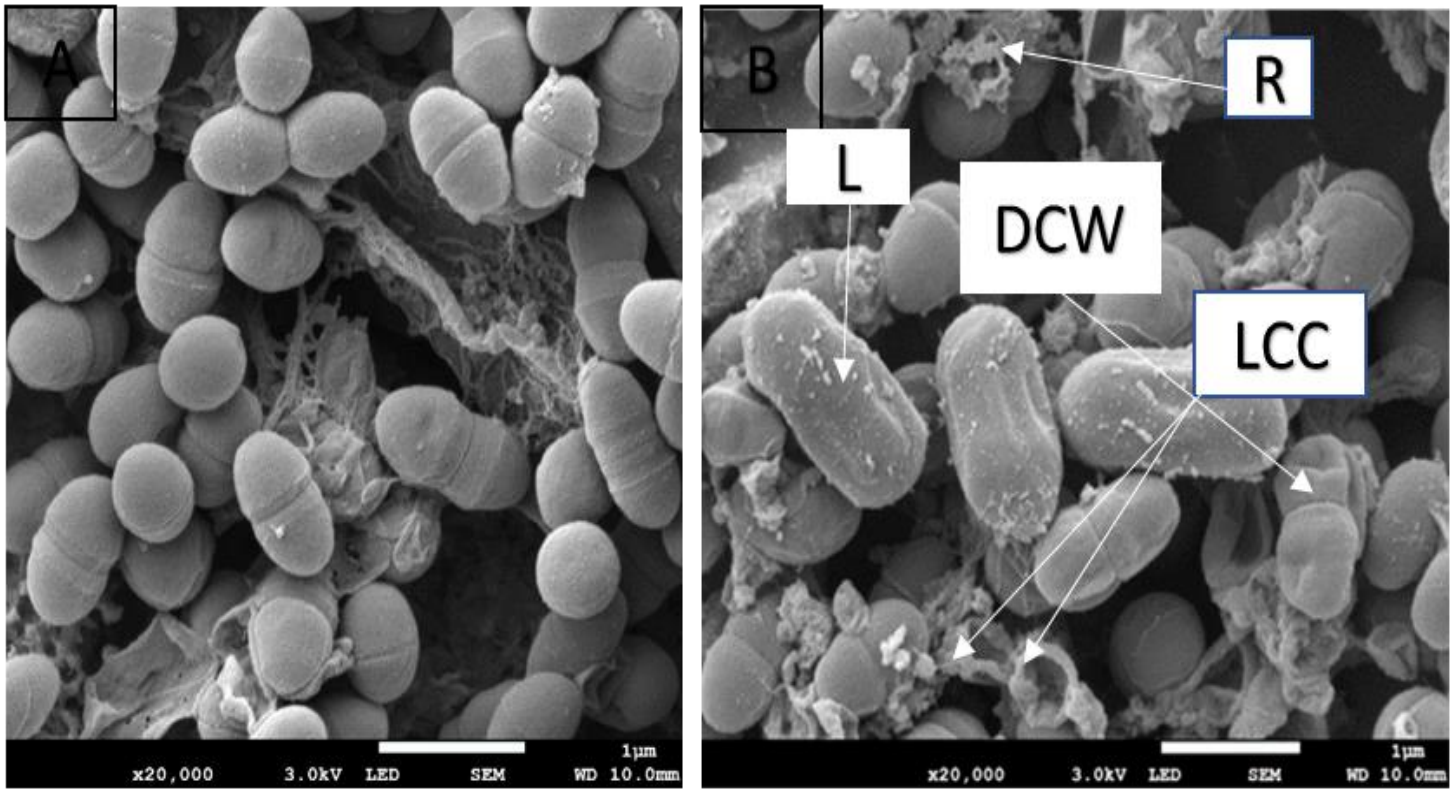

Figure 4.9: SEM image A of a titanium implant showing *S. mutans* control. Image B shows *S. mutans cells* treated with *Salvia officinalis* oil. The image shows loss of cell content (LCC), cell division (CD), damaged cell walls (DCW), elongated cells (L) and roughage (R).

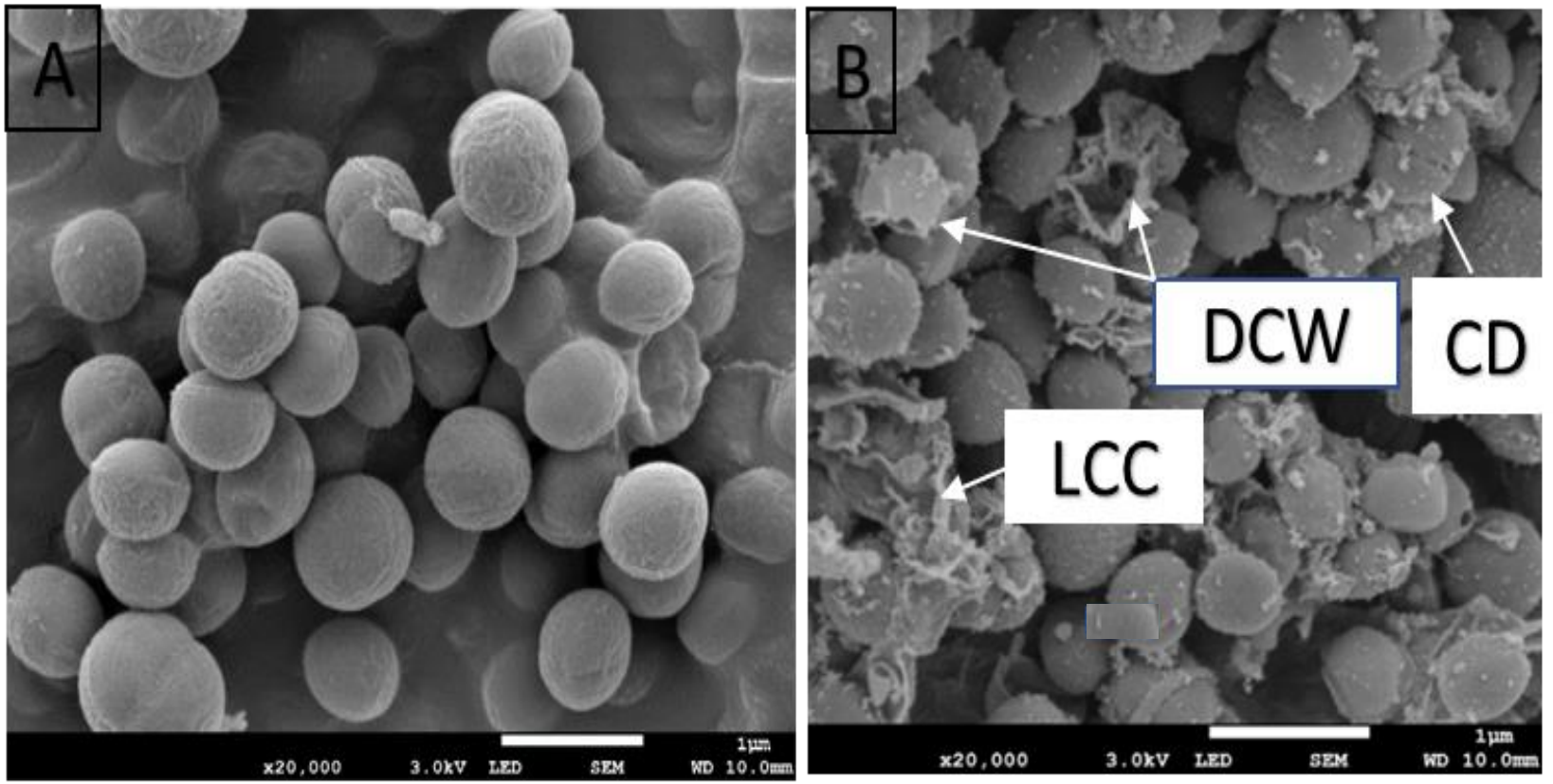

Figure 4.10: SEM image A of titanium implant showing *S. aureus* control cells. Image B shows *S. aureus* cells treated with *Cinnamomum zeylanicum Blume.* Clearly visible are cell division (CD), damage cell walls (DCWs), and loss of cellular content (LCC).

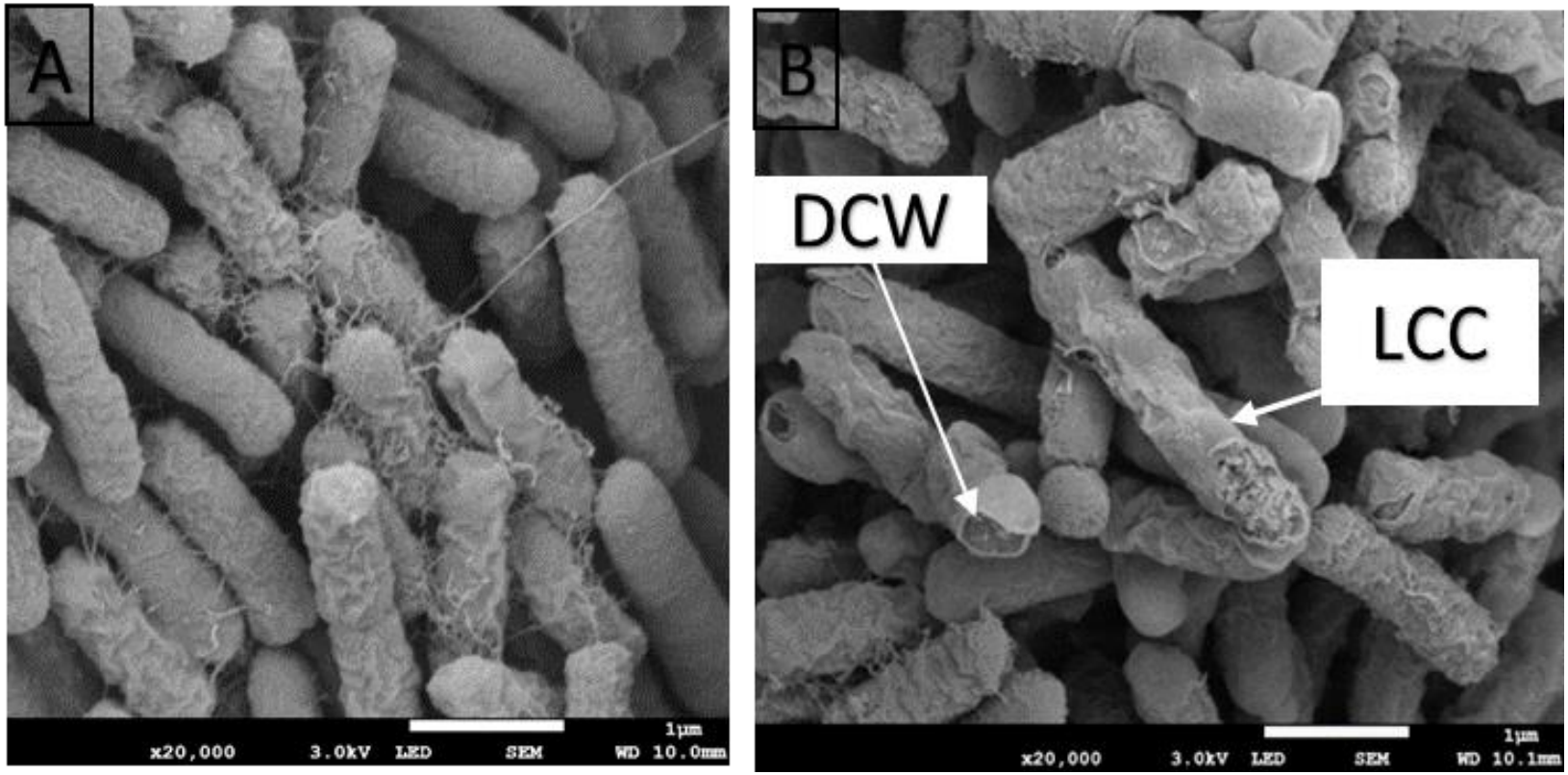

Figure 4.11: SEM image A of titanium implant showing *E. coli* control cells. Image B shows *E. coli* cells treated with *Cinnamomum zeylanicum Blume* oil. Clearly visible are loss of cell content (LCC) and a damaged cell wall (DCW).

Figure 4.10 shows drastic structural changes in *S. aureus* cells caused by their exposure to cinnamon oil. Changes such as cell division, damaged cell walls and loss of cellular content are clearly visible. Moreover, Figure 4.11 shows drastic structural changes caused by cinnamon oil to *E. coli* cells such as loss of cell content and damaged cell walls. Similar results were observed by Cui et al. (2016) due to cinnamon oil that caused cell membrane injury and intracellular material leakage from *Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Salmonella typhi, Klebsiella pneumonia, Pseudomonas aeruginosa and Bacilluspumilus* cells. The antimicrobial activity and structural changes caused by cinnamon essential oil may be due to high concentrations of its major components, namely cinnamaldehyde and eugenol (Raeisi et al., 2015; Zhang et al., 2019). *Cinnamomum zeylanicum Blume* was able to pass through the bacterial cell walls to gain entrance into these cells, causing disruption. Therefore, it may be argued that these essential oils (*Salvia officinalis* and *Cinnamomum zeylanicum Blume officinalis*) have the potential to be used effectively as antimicrobial agents against antibiotic-resistant *S. aureus, S. mutans and E. coli*.

4.6 Chapter Summary

Antimicrobial agents such as essential oils need to be seriously considered as potential antimicrobials in the future because of their mechanism of action against bacterial cells. For instance, bioassay results showed that *Lavendula officianalis, Mentha piperita*, *Cinnamomum zeylanicum Blume, Syzygium aromaticum* and *Salvia officinalis* essential oils may act as effective antimicrobial agents against *Staphylococcus aureus, Streptococcus mutans* and *Escherichia coli*. However, it was interesting to note that *Salvia officinalis* and *Cinnamomum zeylanicum Blume* essential oils showed the most significant inhibitory effects on oral pathogens that had been transferred onto dental implants created for the present work. It was observed that *Cinnamomum zeylanicum Blume* and *Salvia officinalis* essential oils had the ability to pass through the bacterial cell walls to gain entry into the bacteria cells where they caused disruption. Based on these findings, it is important to analyse the chemical components of essential oils as their mode of action depends solely on their chemical components. The main components of the

essential oils were found to be terpenes and phenols that might have been responsible for causing the disturbances on and within these bacteria cells. However, future investigations need to be conducted with specific focus on each chemical component to confirm which may have been responsible for the damage that was observed.

The SEM results clearly showed structural alterations to the cells of the bacteria, such as leakage of the cell contents, cells becoming pleomorphic, irregular cell sizes and some ruptured cells when treated with *Salvia officinalis* and *Cinnamomum zeylanicum Blume* essential oils. The current study thus demonstrated that *Salvia officinalis* and *Cinnamomum zeylanicum Blume* essential oils reduce or inhibit the growth of the *E. coli, S. mutans and S. aureus* bacteria on titanium implants and this further suggests that *Salvia officinalis* and *Cinnamomum zeylanicum Blume* essential oils may be used effectively for the reduction of microbial colonization on the surface of dental implants.

4.7 References

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CHAPTER FIVE

CONCLUSION AND FUTURE STUDIES

CONCLUSION AND FUTURE STUDIES

5.1 Introduction

Oral diseases continue to be a major public health problem in South Africa that needs to be addressed, especially in disadvantaged communities. The most prevalent and significant oral diseases globally are dental caries that are commonly manifest as tooth decay, periodontal disease, tooth loss and cancer of the lips. The bacterial growth in the mouth causes individuals to lose a tooth (or teeth) and therefore dental implantology was introduced to replace the missing tooth. A missing tooth is substituted with a dental implant to which a crown, bridge, or denture can be attached. However, endosseous implants can fail either early or at a later stage of insertion due to implant infection. Periodontitis and peri-implantitis are inflammatory diseases caused by periodontal pathogenic bacteria that destroy the supporting peri-implant tissue. As the bacteria migrate down the surface of the tooth or the implant, the inflammation spreads along with it.

Treatment options such as prophylactic systemic antibiotic regimens have been recommended to minimise an infection after dental implant placement. However, bacteria have become antibiotic resistant due to the over prescription of antibiotics for individuals. Fortunately, natural products such as essential oils have demonstrated their potency to inhibit the growth of drug-resistant microbial strains that are difficult to treat even with conventional antibiotics. Essential oils are used as anti-microbial, anti-diabetic and anti-antioxidant treatment for cancer and cardiovascular diseases. However, there is still limited knowledge regarding the effects of essential oils on the health and well-being of the oral cavity and dental implants. This dearth of knowledge has encouraged the search for new types of effective and nontoxic microbial agents among natural compounds which are found in aromatic plants and which have been used in folk medicine, cosmetics and aromatherapy.

It was against this background that the current study investigated the potency of selected essential oils, namely *Lavendula officianalis, Mentha piperita, Syzygium aromaticum and Salvia officinalis* against oral pathogens namely *Staphylococcus aureus, Streptococcus mutans and Escherichia coli* in the quest to prevent oral pathogens from affecting dental implants. Initially, these five essential oils and their antimicrobial activities were investigated using the bioassay method. The aim was to select the most effective essential oils for the treatment of *E. coli, S. aureus and S. mutans* on the surface of titanium dental implants and to determine their inhibitory effect on bacterial growth on this surface. Of the five essential oils that were tested, *Cinnamomum zeylanicum Blume* and *Salvia officinalis* showed the highest bacterial inhibitory effects. The study thus concludes that these two essential oils that were tested on the surface of titanium implants would be the most effective in the treatment of the bacteria under study.

5.2 Methodology and Key Findings

Various methods were employed to investigate the chemical components of the essential oils and their antimicrobial activities to determine their inhibitory effects on the growth of *E. coli, S. aureus and S. mutans* that are notorious causative agents of dental caries and dental implant failure in the oral cavity.

To investigate the chemical components responsible for inhibiting microbial growth, gas chromatography-mass spectrometry (GC-MS) was used. It was important to analyse the chemical components of each essential oil as the mode of action of essential oils depends mainly on their chemical components (Tariq et al., 2019). It was found that the major compounds of the essential oils were aldehydes and phenols. Phenols are known for their antibacterial activity. The second most common compound of the essential oils was terpene alcohol, which according to Tariq et al. (2019), has the ability to cause disruption to bacteria cells.

This above process was followed by the application of the quantitative microbial bioassay method to test the biological activity of the selected essential oils to determine their ability

to inhibit bacteria growth. Initially the purpose was to determine which essential oil inhibited bacterial growth best. It was found that *Salvia officinalis and Cinnamomum zeylanicum Blume* contained the most effective essential oils as they demonstrated significant inhibitory effects based on their respective inhibition diameters. These two essential oils reacted differently on each bacterium. For instance, *Cinnamomum zeylanicum Blume* showed the highest inhibition effect on S. *aureus* (40 mm inhibition diameter) and *E. coli (*45 mm inhibition diameter), whereas *Salvia officinalis* showed the highest inhibition on *S. mutans* (40 mm inhibition diameter).

Following the above assessment process, microdilution assay was performed. *Cinnamomum zeylanicum Blume* and *Salvia officinalis* essential oils were selected for the microdilution assay investigation because of their significant inhibitory effects on *Staphylococcus aureus, Streptococcus mutans and Escherichia coli*. *Cinnamomum zeylanicum Blume* essential oil showed a MIC of 10 µg/ml for treating *S. aureus* and 20 µg/ml for treating *E. coli,* while *Salvia officinalis* essential oil showed 5 µg/ml for treating *S. mutans* cells. Furthermore, on the surface titanium implant samples *Cinnamomum zeylanicum Blume* oil also showed a MIC of 25 µg/ml for treating *S. aureus* and 50 µg/ml for treating *E. coli,* while *Salvia officinalis essential oil* showed 12.5 µg/ml for *treating S. mutans* cells.

Scanning electron microscopy (SEM) was used to assess the structural changes that had occurred on and within the bacteria cells (*S. aureus, E. coli* and *S. mutans*) due to the essential oil activity of *Cinnamomum zeylanicum Blume* and *Salvia officinalis.* The essential oils were tested at their minimum concentrations (MICs). The preparation of the cells for analysis using SEM was carried out according to the protocol proposed by Ncango et al. (2010).

Based on the findings, it was concluded that, due to the noticeable inhibition zones, minimum inhibition concentrations of *Cinnamomum zeylanicum Blume* and *Salvia officinalis* caused drastic structural changes to the bacterial cells. Their action against the selected oral pathogens was thus deemed impressive evidence of their antimicrobial

effectiveness and for this reason it may be argued that these essential oils should be considered as potential antimicrobial agents in the dentistry field. The finding that these two essential oils acted differently on each bacterial cell may be attributed to their chemical components, but this should be further investigated.

5.3 Future Studies

The current study revealed the following areas that should be investigated in future studies:

- Determining the extent to which essential oils can adhere to the surface of titanium dental implants;
- Determining whether *Cinnamomum zeylanicum Blume* and *Salvia officinalis* interfere with osseointegration when titanium implant materials are coated with their essential oils;
- Determining whether these essential oils could harm or kill human cells when titanium implants are coated with them;
- Assessing the life expectancy of essential oils on titanium implant materials;
- Investigating the potential use of essential oils as disinfectants on the surfaces of dental tools;
- Assessing the use of *Cinnamomum zeylanicum Blume* and *Salvia officinalis* in mouth rinses to combat *Staphylococcus aureus, Streptococcus mutans* and *Escherichia coli;* and
- Testing each chemical component in these essential oils to confirm which chemical component is responsible for damaging bacterial cell walls and the cell contents.

5.4 References

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