Efficacy of *Melaleuca alternifolia* and *Pelargonium graveolens* against *Staphylococcus aureus* and *Staphylococcus epidermidis* associated with knee implants

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STATEMENT OF INDEPENDENT WORK

I, Ntombokhanyo Mbanjwa, student number _____, herewith proclaim that this submitted study project to the Central University of Technology, situated in Free State for MASTER OF HEALTH SCIENCE degree: ENVIRONMENTAL HEALTH consists of my own work additionally, it hasn't been submitted to any institution before by me or anyone else in order to meet the requirements for obtaining of any kind qualification.

Signature of student **Date**

…… ……

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SUMMARY

Knee replacement surgery is experiencing a surge in demand worldwide; however, knee infections represent a substantial public health threat, particularly for underserved communities. While knee implantology was innovated to restore functionality to damaged knees, the risk of infections due to bacterial colonisation around the implant remains a serious concern. The overuse of antibiotics not only jeopardises patient health but also escalates healthcare costs. Although prophylactic antibiotic regimens are employed to mitigate infection risks, the emergence of antibiotic resistance due to excessive utilisation has complicated this approach. In this context, essential oils emerge as a promising solution to combat these challenges.

Essential oils, known for their natural origins, serve as potent antibacterial agents due to their mechanisms of action, which include disrupting cell walls, damaging cytoplasmic membranes, and causing cytoplasmic coagulation. This study specifically investigates the Essential oils' effectiveness in combatting bacteria associated with knee implants. The research examines the bactericidal properties of *Melaleuca alternifolia* (tea tree essential oil) and *Pelargonium graveolens* (geranium essential oil) essential oils against the antibiotic-resistant strains *Staphylococcus epidermidis* and *Staphylococcus aureus*. The findings suggest that both *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils effectively inhibit bacterial growth linked to knee implants, thereby potentially reducing the necessity for knee implant revisions.

The essential oils' chemical constituents were examined utilising gas chromatography- mass spectrometry (GC-MS).Terpinen-4-ol was discovered to be a key component of *Melaleuca alternifolia* essential oil, including α-pinene, γ-terpinene, limonene, and cymene, which are associated with antibacterial and anti-inflammatory properties. *Pelargonium graveolens* essential oil consists of citronellol and geraniol as major components that contribute to cell wall disruption, loss of cellular contents, and overall bacterial deformation. The research employed bioassay methods to evaluate the antibacterial effects of *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils against *S. aureus* (ATCC 25923) and *S. epidermidis* (ATCC 12228). Both *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils demonstrated significant inhibitory effects

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on the growth of *S. aureus* and *S. epidermidis*. To ascertain the concentration at which these essential oils can inhibit microbial growth, the minimum inhibitory concentration (MIC) method was utilised. Additionally, the study sought to examine the structural transformations induced by essential oils in microorganisms, employing scanning electron microscopy (SEM) and transmission electron microscopy (TEM) techniques. The SEM results revealed observable morphological changes, including slight roughness (R) of the cell surfaces, loss cellular content (LCC), and damage to the cell walls (DCW). The TEM results revealed drastic alterations in the cellular structure of *S. aureus* and *S. epidermidis* following exposure to *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils. Notable changes included damaged cell walls, the formation of holes, and the depletion of cellular contents, ultimately resulting in cell death.

This research holds significant implications for the use of natural products like essential oils as potential alternative bacteriostatic agents. During microbial assessments, it was observed that *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils compromised the cell membranes of *S. aureus* and *S. epidermidis*, leading to cell death. Consequently, this indicates that both essential oils can effectively target multiple bacterial sites, particularly the plasma membrane and cytoplasm. Moreover, the study highlights some advantages of using natural products such as *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils, including the need for the healthcare sector to reduce reliance on antibiotics and consider essential oils as viable alternatives in the fight against microorganisms responsible for knee infections, ultimately decreasing the need for revision surgeries.

However, despite the promising nature of essential oils as alternatives to antibiotics, further research is imperative. The impact of essential oils on health and integrity of knee implants, the optimal concentrations that ensure bacterial eradication without harming human cells, and the duration of essential oil adherence to implants remain areas requiring additional exploration, as well as investigating the biofilm formation of bacterial strains on the experimental knee implant.

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CHAPTER ONE: BACKGROUND

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THE USE OF *PELARGONIUM GRAVEOLENS* AND *MELALEUCA ALTERNIFOLIA* ESSENTIAL OILS AGAINST *STAPHYLOCOCCUS AUREUS* AND *STAPHYLOCOCCUS EPIDERMIDIS* ASSOCIATED WITH KNEE IMPLANTS

1.1. Background

Knee implant infections represent a significant global health issue and are primarily caused by a range of bacteria such as coagulase-negative staphylococci and *Staphylococcus aureus* strains, other Gram-positive cocci, anaerobes, and fungi (Duque *et al.,* 2023). The management of these infections, particularly those caused by *S. aureus* and *S. epidermidis*, has become increasingly challenging due to the development of antibiotic resistance (Duque *et al.,* 2023). This urgent situation necessitates the exploration of alternative treatments, with numerous researchers advocate.ng for the use of natural products, such as essential oils. These oils have demonstrated antiviral, antimitotic, antitoxigenic, and antiparasitic properties and have proven effective against various bacterial species, including those resistant to conventional antibiotics (Setlhare, 2017; Kariminik *et al*., 2019).

Comprehensive reviews published by Gajdács (2019) and Boren *et al*. (2020) highlighted the Centers for Disease Control and Prevention's (CDC) classification of methicillin-resistant *S. aureus* (MRSA) as a critical drug-resistant threat since 2013. The reviews indicated that MRSA infections lead to over 80,000 invasive cases and more than 11,000 deaths annually in the United States. MRSA's propensity for bloodstream metastases can result in serious conditions such as osteomyelitis and endocarditis, contributing to alarmingly high mortality rates of 30% and 50% at 30 and 90 days, respectively. The broader implications of antibiotic resistance are profound, claiming over 35,000 lives in the US each year (Boren *et al*., 2020). In South Africa a decrease in MRSA prevalence from 36% in 2006 to 24% during 2007-2011 ocured due to the implementation of infection control policies that are effective. Despite this success, many African nations still report MRSA rates lower than 50%, although these rates have seen an increase since 2000.

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Ejaz *et al*. (2023) noted that vancomycin, once the preferred treatment for MRSA infections since the late 1980s, has seen *S. aureus* develop resistance against it, posing a significant public health risk (Kest and Kaushik, 2019; Ejaz *et al.,* 2023). While several studies from South Asia, including Bangladesh, Sri Lanka, India, Pakistan, and Nepal, have documented decreased sensitivity and complete resistance to this antibiotic, there is a notable absence of research from Afghanistan, the Maldives, and Bhutan regarding non-susceptible *S. aureus* strains (Setlhare, 2017; Kariminik *et al*., 2019; Ejaz *et al.,* 2023). A recent systematic review indicated a higher prevalence of vancomycin-resistant *S. aureus* (VRSA) in Asia (1.2%), Europe (1.1%), America (3.6%), and Africa (2.5%) (Ejaz *et al*., 2023).

Since their discovery, antibiotics have primarily served human health needs; their application has expanded to include animal medicine, agriculture, and food security (Manyi-Loh, 2018). Unfortunately, the widespread and often inappropriate use of antibiotics has led to the emergence of resistant microorganisms, representing a significant threat to global health and making the treatment of microbial infections increasingly complex and costly (Manyi-Loh, 2018).

Consequently, there is an urgent need for novel strategies to combat infections. Traditional and folk medicine have long utilised natural plant-derived products, including essential oils, which are volatile substances extracted from aromatic plants with promising potential as new natural medications (Li *et al.,* 2020). Essential oils, consist of tens to hundreds of secondary metabolites that primarily include phenylpropanoids and terpenoid derivatives, which are able to stop or slow down the growth of bacteria, yeasts and moulds. Essential oils and their components have activity against a variety of targets, particularly the membrane and cytoplasm, and in some cases, they completely change the morphology of the cells (Nazzaro *et al*., 2013; Magunga, 2016) . The most common components in these volatile oils are sesquiterpenes, phenolic compounds, and both oxygenated and non-oxygenated monoterpenes (Lupia *et al.,* 2024).

Due to their remarkable biological and medicinal properties, such as bactericidal, insecticidal, antiparasitic, virucidal, fungicidal, analgesic, spasmolytic, anaesthetic, antioxidative, and anti-inflammatory qualities, the application of essential oils in nanotechnology is expanding (Renuka, 2017). Recent research suggests that essential oils may alter bacterial membrane permeability,

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impacting both transport systems and energy production, although the exact mechanisms underpinning their antibacterial action remain incompletely understood. Given that resistance to essential oils has not been established, it is hypothesised that their multi-component composition contributes to their broad-spectrum antibacterial activity (Bunse *et al*., 2022).

This research will explore the effectiveness of *Melaleuca alternifolia* essential oil and *Pelargonium graveolens* essential oils against antibiotic-resistant strains of *S. aureus* and *S. epidermidis* associated with knee implants.

1.2. Antibiotic Resistance Tactics Employed by Bacteria

In medical settings, many bacterial pathogens can endure and proliferate when there are antibacterial agents present that are typically effective against their specific type (Setlhare, 2017). To ensure their survival and dominance, bacterial species adapt to host defence mechanisms and interfere with crucial processes necessary for the effective action of antimicrobial agents. For instance, certain Gram-negative bacteria, such as *Enterobacter aerogenes* and *Pseudomonas aeruginosa*, modify the frequency, size, and selectivity of cell membrane porin channels, thereby reducing antibiotics intake, including aminoglycosides and beta-lactams (Nazzaro *et al*., 2013; Setlhare, 2017). When bacteria can obstruct access to penicillin-binding proteins (PBPs) and ribosomes—primary targets of beta-lactams and aminoglycosides, respectively—they enhance their chances of survival in the presence of antibiotics.

Another strategy employed by bacteria to resist antibiotics involves Antimicrobial compounds being expelled from the cell by specialised or general efflux pumps (Setlhare, 2017; Chinemerem Nwobodo *et al*., 2022). For example, complete proteins found in bacteria like *E. coli, Bacillus cereus*, *S. aureus*, and *S. pneumoniae* function as efflux pumps, facilitating the removal of certain drugs from the cell (Chinemerem Nwobodo *et al*., 2022). Moreover, specific efflux pumps are known to actively discharge certain antibiotics, including tetracycline, macrolides, streptogramins, and lincosamides (Chinemerem Nwobodo *et al*., 2022).

Bacteria also utilise enzymatic deactivation as a means of protecting themselves from antibiotics, as seen in the action of the enzyme beta-lactamase, which hydrolyses the beta-lactam ring in

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penicillin and cephalosporin (Wright, 2005; Hamilton and Wenlock, 2016). Inactivation of penicillin prevents binding to PBPs, thus preserving cell wall synthesis. Additionally, bacteria have the capability to modify or conceal essential target sites to evade detection, inhibiting further binding or action of active antimicrobial agents (Wright, 2005; Hamilton and Wenlock, 2016).

The evolution of significant antibiotic resistance mechanisms among bacteria has catalysed an increased interest in exploring alternative therapies for bacterial infections, particularly in human populations. Essential oils represent one such alternative, with the potential to serve as effective antimicrobial agents against infectious diseases that pose grave risks to patients, especially those with compromised immune systems (Voon *et al*., 2012; Swamy *et al*., 2016). As noted by Chinemerem Nwobodo *et al*. (2022) in Figure 1.1, bacterial strains must possess the capability to interfere with one or more crucial processes necessary for effective activity of antimicrobial agents to survive exposure to antibiotics. Bacterial species typically adopt one of four fundamental survival strategies (Chinemerem Nwobodo *et al*., 2022), underscoring the necessity for exploring alternatives such as natural products and essential oils rather than relying solely on antibiotics.

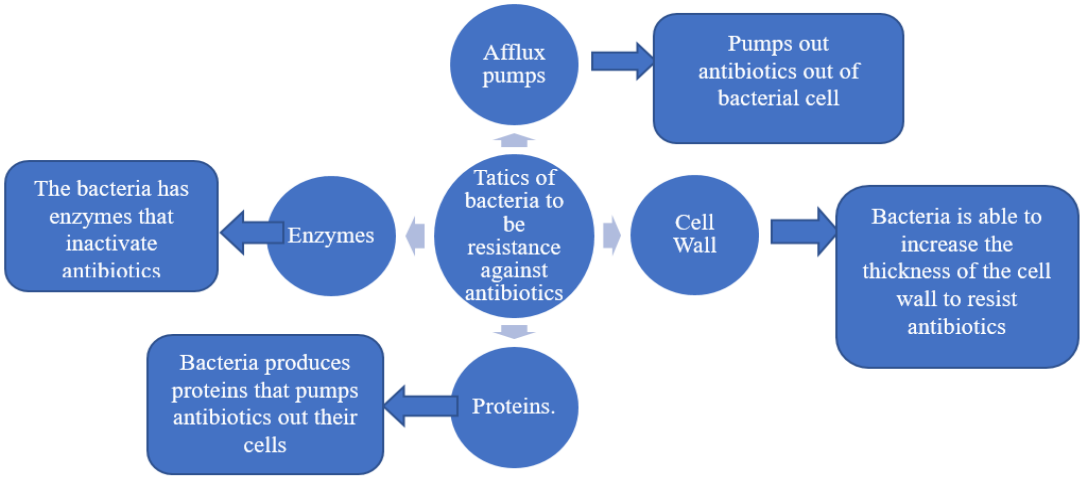


Figure 1.1: Tactics of bacteria to be resistant against antibiotics (Chinemerem Nwobodo *et al*., 2022)

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1.3. Essential Oils

Essential oils are aromatic, oily liquids extracted from various plant materials, including buds, roots, flowers, wood, leaves, bark, fruits, twigs, and seeds (Reddy, 2019; Duque *et al*., 2023). Commonly referred to as ethereal or volatile oils, essential oils are typically obtained through methods such as extraction, effleurage, fermentation, or expression; however, steam distillation is the most widely adopted technique for commercial production (Duque *et al*., 2023). Unlike fixed oils, like olive oil, essential oils possess specific therapeutic properties beneficial for enhancing patient Well-being and health in clinical environments (Cimino *et al*., 2021). Essential oils are volatile, evaporating when exposed to air, and have been utilised to address various health conditions, including cancer, diabetes, Alzheimer's disease, and cardiovascular diseases. The oils exhibit antiviral, antimitotic, antitoxigenic, and antiparasitic properties, effectively targeting a diverse range of bacterial species, including those resistant to antibiotics (Ntondini and Lenetha, 2022; Duque *et al*., 2023).

1.3.1. Essential Oils as Potential Antimicrobials

In developing nations, illnesses that spread pose significant health challenges that can lead to fatal outcomes if not managed with appropriate antimicrobial agents (Ojeda-Thies *et al*., 2020; Garvin *et al*., 2023). Researchers worldwide are actively seeking new antimicrobial agents effective against the persistent emergence of antibiotic-resistant bacterial pathogens (Terreni *et al.*, 2021). In response, scholars have begun to focus on essential oils, recognising their ability to effectively target multiple sites within a variety of organisms (Setlhare, 2017; Asma *et al*., 2022). The growing interest in natural products, such as essential oils, is driven by the perception that they are safer and more reliable alternatives to synthetic drugs, which may have adverse effects (Ntondini and Lenetha, 2022).

Essential oils consist of intricate blends of secondary metabolites composed of ten to twelve molecules classified primarily as phenylpropanoids and terpenoid derivatives, along with various combinations of monoterpenes and sesquiterpenes (Wani *et al*., 2021; Allizond *et al*., 2022). These oils include a wide variety of substances, like oxides, sulfur, and fatty acid derivatives. The phenylpropanoid and terpenoid families represent the primary constituents of many essential oils,

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accounting for approximately 85% of their overall composition (Setlhare, 2017; Stringaro *et al.*, 2018). Notable plant families containing essential oils include Apiaceae, Lamiaceae, Zingiberaceae, Lauraceae, Asteraceae, Myrtaceae, Rutaceae, and Cupressaceae (Moghaddam and Mehdizadeh, 2017; Setlhare, 2017). Essential oils like clove, peppermint, lemon, tea tree, and cinnamon have been employed to promote wound healing due to their antioxidative and anti-inflammatory properties, as well as their capacity to inhibit fungi, viruses, and bacteria (Allizond *et al*., 2022; Sharma *et al*., 2023).

1.3.2. Mode of Action of Essential Oils Against Microorganisms

The mechanisms through which essential oils exert their effects on microorganisms involve three primary facets: (1) the alteration of microbial morphology by interactions with enzymes membrane, leading to a loss in firmness of cell wall and integrity; (2) changes in cell membrane permeability, cytoplasmic granulation, and membrane rupture; and (3) the existence of hydroxyl (OH) groups that form hydrogen bonds with enzymes, subsequently altering various intracellular functions (Magunga, 2016; Setlhare, 2017; Angane *et al*., 2022). Essential oils' capacity to enter microbiological cell and change their composition and operation results in significant impacts on intracellular processes. For example, essential oils can disrupt the electron transport chain and modify membrane permeability (Magunga, 2016; Setlhare, 2017; Angane *et al*., 2022).

Disruption of the electron transport mechanism can lead to cell lysis and death, as it halts the synthesis of essential cellular components, disrupts the motive force of the proton, and impedes protein translocation (Angane *et al*., 2022). Furthermore, essential oils have the potential to decrease intracellular ATP concentrations, which is connected to degradation in the membrane of microorganisms (Rao *et al.,* 2019; Angane *et al*., 2022). Additionally, specific compounds found in essential oils may interfere with the proteins that are involved in the transport of vital substances into cells, resulting in instability of the phospholipid bilayer, compromising the role of the plasma membrane, reduction of vital intracellular elements, and inactivation of enzymatic processes (Magunga, 2016; Setlhare, 2017; Angane *et al*., 2022).

Moreover, the barrier of permeability supplied by bacterial cell membranes facilitates a range of cellular functions, including solute transport, metabolic regulation, and energy preservation

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(Nazzaro *et al*., 2013; Magunga, 2016). The cell membrane also plays a crucial role in maintaining turgor pressure (Magunga, 2016). The Essential oils' harmful impact on membrane structure may account for some of their antibacterial properties. Indeed, the cytoplasmic membrane, cell wall, cytoplasm coagulation, membrane protein damage, and increased permeability, which causes the contents of cells to leak, are among the mechanisms through which essential oils operate (Verdeguer *et al.,* 2020; Angane *et al*., 2022). This interruption of intracellular ATP pool ultimately reduces the proton motive force (Mani-López *et al.*, 2021; Angane *et al*., 2022) (see Figure 1.2).

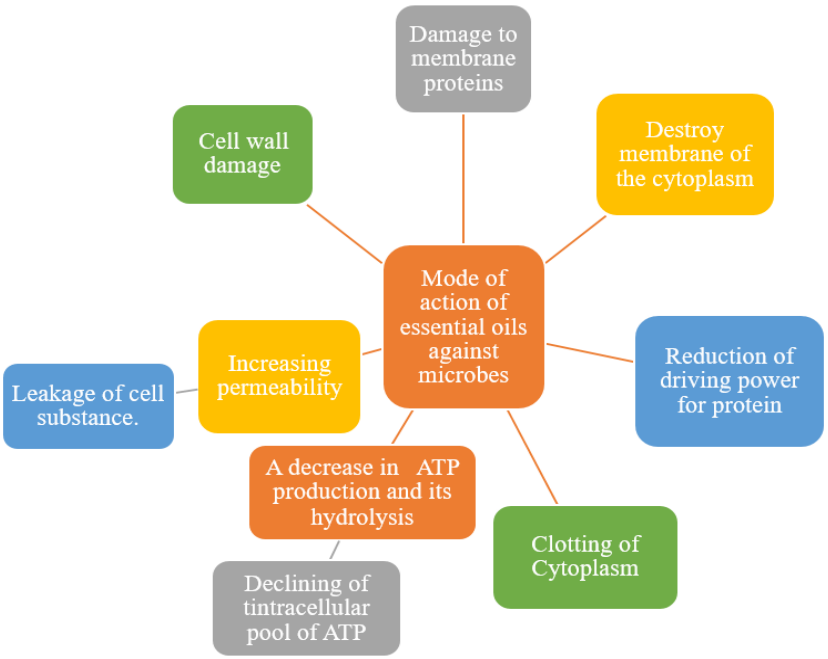


Figure 1.2: Mode of action of the essential oils in opposition to antibiotic-resistant bacteria

These three features are interconnected, as the hydrophobic nature of these compounds permits them to pass through cell membranes and interact with intracellular components, thereby impacting membrane integrity and intracellular enzyme activity (Nourbakhsh *et al*., 2022). Certain hydrophobic compounds like terpenes, terpenoids, phenols, and alcohols found in essential oils can modify microbial membrane permeability to cations like K+ and H+, affecting flow of proton, altering intracellular pH, and influencing the overall chemical makeup and cellular function

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(Nazzaro *et al*., 2013; Magunga, 2016; Nourbakhsh *et al*., 2022). The capacity of hydrophobic substances to separate into the cytoplasmic membrane's lipid phase is crucial for their antibacterial efficacy; nevertheless, it's crucial to remember that higher solubility does not always correlate with increased antibacterial activity (Magunga, 2016).

1.4. Rationale

1.4.1. Problem Statement

Knee implants are frequently utilized to substitute damaged joints; nonetheless, the introduction of foreign materials heightens the potential of bacterial adherence, especially from *staphylococci*, which account for a considerable percentage of prosthetic joint infections (PJIs) (Schmidt, 2020; Dawood *et al*., 2021). Notably, *Staphylococcus aureus* accounts for 22–23.6% and *Staphylococcus epidermidis* for 19–37.5% of these infections. The development of biofilms on implant surfaces complicates therapy, since bacteria within these formations have diminished metabolic activity, heightened resistance to antimicrobial drugs, and increased pathogenicity due to the protective extracellular matrix they generate (Rosato *et al*., 2020). Consequently, biofilm-associated infections present significant therapeutic hurdles, resulting in heightened hospital admissions and treatment failures(Haenle *et al*., 2012). The rise of drug-resistant bacteria worsens this public health risk, emphasizing the urgent need for novel ways to address implant-related infections (Urish *et al*., 2013; Shah *et al*., 2020).

To tackle this issue, researchers have turned to the antibacterial characteristics of essential oils (EOs) obtained from several medicinal plants (Rosato *et al*., 2020). EOs, which are concentrated aromatic extracts, have been demonstrated to exhibit different bioactive chemicals with antioxidant, antibacterial, and anti-inflammatory activities (Magunga, 2016; Setlhare, 2017). Furthermore, EOs, such as *Pelargonium graveolens* and *Melaleuca alternifolia*, can effectively inhibit the growth of antibiotic-resistant bacteria commonly associated with knee implants, thereby offering a promising alternative to conventional antibiotics (Schmidt, 2020; Dawood *et al*., 2021). The principal components of *Pelargonium graveolens* oil, including geraniol and citronellol,

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contribute to its antimicrobial efficacy, while *Melaleuca alternifolia* oil, rich in terpinen-4-ol, is recognised for its potent antibacterial and antiseptic properties (Bruellhoff *et al*., 2010).

Despite comprehensive studies on the antibacterial properties of these essential oils in many worldwide settings, a significant deficiency persists in their assessment on the African continent. (Schmidt, 2020; Dawood *et al*., 2021). Thus, this study seeks to address this gap by characterizing *Pelargonium graveolens* and *Melaleuca alternifolia* oils via gas chromatography–mass spectrometry (GC-MS), evaluating their antimicrobial efficacy against *S. aureus* and *S. epidermidis* through a bioassay preparation method and minimum inhibitory concentrations, and investigating their impact on bacterial morphology using scanning and transmission electron microscopy.

This study not only helps to understand the potential uses of EOs in reducing implant-related infections, but it also prepares the road for their incorporation into orthopaedic materials, which might improve the clinical efficacy of knee implants.

1.4.2. Study Aim

The primary aim of this study is to evaluate the effectiveness of *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils against the ATCC strains of *S. epidermidis* and *S. aureus* associated with titanium knee implants.

1.4.3. Objectives of the Study

To achieve the overall aim of this research, the following objectives have been established:

- To investigate the chemical constituents of *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils using gas chromatography-mass spectrometry (GC-MS)
- To determine the antimicrobial activity of these essential oils against antibiotic-resistant bacteria through bioassay preparations
- To assess the minimum inhibitory concentration (MIC) at which *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils inhibit the growth of *S. aureus* and *S. epidermidis*

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- To examine the structural changes induced by *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils on bacterial cell walls using transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

1.5. Chapter Summary

The failure of knee implants is predominantly attributed to the prevalence of bacterial biofilm infections. The development of bacterial biofilms on the titanium surfaces of knee implants can lead to illnesses that occur around implants which adversely impact the sustained success of these implants when left untreated. While biomaterials are employed to replace damaged knee components, their effectiveness is compromised if appropriate antimicrobial agents are not utilised, as bacteria will colonise the implant's surface. Therefore, it is crucial to prevent biofilm formation on the surfaces of biomaterials to avert the spread of pathogens and the degradation of these materials. Products made naturally, like essential oils, are recognised as potential agents that fight bacteria and can be employed to prevent the growth of biofilms on titanium implant surfaces, ultimately preventing knee infections. The emergence of antibiotic-resistant microorganisms presents a significant challenge in public health; hence, this research is focused on the utilisation of essential oils to combat antibiotic-resistant microorganisms associated with knee implants.

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CHAPTER TWO:
LITERATURE REVIEW

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EXPLORING THE EFFECTIVENESS OF *PELARGONIUM GRAVEOLENS* AND *MELALEUCA ALTERNIFOLIA* ESSENTIAL OILS AS NATURAL REMEDIES AGAINST PATHOGENS RELATED TO KNEE INFECTIONS

2.1. Introduction

Human life expectancy has increased significantly, and this enhancement in longevity must be accompanied by an improved quality of life, particularly for senior citizens. However, as one ages, the body's capacity to promote natural recovery following injuries diminishes, even among those who maintain a healthy lifestyle (Allizond *et al*., 2022). Additionally, younger individuals increasingly rely on surgical interventions to treat injuries sustained during sports or accidents. Consequently, the rising number of surgical procedures has led to an increased demand for implants (Klug *et al*., 2021; Allizond *et al*., 2022). The utilization of bone grafts across a range of medical specialties increases this need even further, including oncology, spinal surgery, and traumatology (Lin *et al.*, 2017; Hasan *et al*., 2018; Allizond *et al*., 2022). Conditions such as severe trauma, rheumatologic diseases, osteoarthritis, irreparable human tissues, and tumour resections prevalent among the elderly can result in irreversible defects that do not heal (Zardi *et al*., 2020; Allizond *et al*., 2022).

Orthopaedic devices are required to replace damaged or missing joints and to facilitate tissue recovery in various regions of the body (Comini *et al*., 2022). However, the implant structure, due to its proximity to biological fluids, provides a perfect setting for the attachment and proliferation of bacteria and fungi, leading to significant infections, impaired implant functionality, and the creation of an inflammatory environment (Comini *et al*., 2022; Allizond *et al*., 2022). Orthopaedic infections are predominantly caused by diverse bacterial species, notably coagulase-negative *staphylococci* and Gram-positive cocci such as *S. aureus*, which can lead to implant failure (Comini *et al*., 2022).

Implant failure can occur at both early and late stages; early failure typically happens before implantation, while late failure can occur years after placement (Wignadasan *et al*., 2023).

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Pathogenic bacteria often colonise implant surfaces, resulting in failures and damage to peri-implant tissues. In Figure 2.1, biofilms, which consist of bacterial communities that adhere to surfaces, form on knee implants after surgery and increase the likelihood of infection from skin-associated pathogens. The biofilm surrounding the implant can lead to knee irritation and inflammatory conditions (Wignadasan *et al*., 2023).

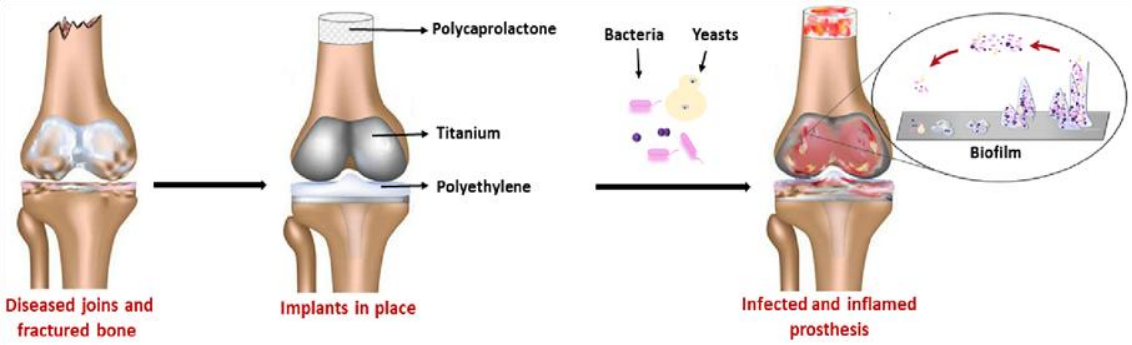


Figure 2.1. Diagram demonstrating knee infection caused by biofilm infestation (Allizond *et al*., 2022)

Over half a million types of medical devices, including knee implants, dental implants, and catheters, are reportedly marketed for medical purposes worldwide (Arciola *et al.*, 2018; Cao *et al.*, 2022). The number of procedures involving Implanted medical equipment is anticipated to rise significantly in the upcoming years due to the growing elderly population. It is estimated that by 2030, there will be 1.26 million total knee replacement procedures carried out in the United States, representing an increase of 85% (Sloan *et al.*, 2018; Cao *et al.*, 2022). In Germany, the quantity of total knee replacements techniques is anticipated to increase by 45%, exceeding 244,000 treatments by 2040 (Rupp *et al*., 2020). Bacterial infections remain one among the most common and serious issues related to the clinical use of medical devices that are implantable (Arciola *et al.*, 2018; Cao *et al.*, 2022). In Europe, over 33,000 deaths are attributed annually to antibiotic-resistant bacteria, with approximately one-third of these fatalities occurring in Italy (Cassini *et al.*, 2019; Iseppi *et al*., 2021). Italy ranks among the highest consumers of antibiotics for both human and veterinary purposes and exhibits significant resistance to antibiotics among key pathogens (*Enterococcus faecalis*, *S. aureus*, *Escherichia coli*, etc.) (Iseppi *et al*., 2021). The increasing

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resistance to nearly all antibiotic classes complicates treatment efforts and underscores the urgent need for novel therapies to address this public health challenge (Iseppi *et al*., 2021).

S. aureus and *S. epidermidis* are among the most common infectious agents implicated in knee infections. These species are part of the normal skin flora in humans and can form biofilms on prosthetic devices (Trobos *et al*., 2022). *S. aureus* is frequently isolated from knee infections at surgical sites (Francois *et al*., 2018). While antibiotics are typically employed to nurse bacterial illnesses, the emergence of biofilms and increasing antibiotic resistance often leads to treatment failures (Francois *et al*., 2018).

One of the additional challenges promoting the resistance to antibiotics is the over-the-counter availability of antibiotics in several countries. Patients often do not adhere to prescribed dosages, resulting in the development of resistance (Atshan *et al*., 2023). Mumu and Hossain (2018) also noted that the inappropriate use of antibiotics contributes to resistance, as individuals seeking treatment for viral illnesses such as colds or flu are frequently prescribed ineffective antibiotics (Mumu and Hossain, 2018; Atshan *et al*., 2023). Considering the growing prevalence of antibiotics-resistant bacteria, it is essential to explore alternative treatment options, such as essential oils.

Essential oils exhibit considerable therapeutic potential due to their ability to extract active compounds from herbs and flowers. These volatile substances are particularly effective in combating microbes, such as viruses, fungus, and bacteria (Mumu and Hossain, 2018). Their multifaceted properties—like anti-inflammatory, antiviral, and antimicrobial properties—along with their immune-boosting effects, have been extensively documented by researchers (Mumu and Hossain, 2018; Atshan *et al*., 2023). It is widely acknowledged that many antibiotics, including penicillin and ciprofloxacin, have become ineffective, rendering some infections untreatable (Atshan *et al*., 2023). Consequently, it is essential to find alternatives to antibiotics, including the exploration of natural resources like essential oils (Mumu and Hossain, 2018). Essential oils have diverse applications across various sectors, including their use as preservatives and additives in food products, fragrances in cosmetics, and therapeutic agents in pharmaceuticals (Prakash *et al*., 2018).

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Consequently, there has been a growing trend among health-conscious consumers towards the utilisation of natural and organic products. The essential oil market worldwide was estimated to be worth 226.8 kilotons in 2018, with a projected compound annual growth rate (CAGR) of 8.6% from 2019 to 2025 (Irshad *et al*., 2020). The antibacterial properties and health benefits of essential oils have spurred increased demand worldwide. Unlike conventional antibiotics, there is currently no evidence of essential oils' ability to combat germs, which have been historically effective against various microorganisms. Thus, it is imperative to investigate the potential of essential oils as antibacterial agents in knee arthroplasty, given their longstanding use and efficacy against pathogens.

2.2. Biomaterials

Biomaterials refer to substances applied in the fabrication of prosthetics and biomedical devices intended to replace or repair damaged bodily functions, with the ultimate goal of preserving or enhancing life (Gobbi *et al*., 2020). Common types of biomaterials include metals, ceramics, and polymers, each utilised to restore or maintain bodily function (Ebrahimi, 2018; Gobbi *et al*., 2020). Titanium and its alloys have gained popularity due to their improved mechanical qualities, biocompatibility and resistance to corrosion, making them highly favoured in medical applications (Gobbi *et al*., 2020).

The biomaterials field research has focused on investigating various materials for the replacement or regeneration of damaged or degenerative tissues and organs over the past fifty years (Gobbi *et al*., 2020). In the United States alone, nearly 13 million prosthetic limbs and medical devices are implanted annually (Wang, 2013; Gobbi *et al*., 2020). Biomaterials are utilised in various components of the human anatomy, such as implants, blood vessel stents, and artificial heart valves used in the shoulders, hips, knees, elbows, ears, and orthodontics (Gobbi *et al*., 2020). It is estimated that metal biomaterials account for over 70% to 80% of implant materials, which are essential for reconstructing damaged tissues, particularly hard tissues (Gobbi *et al.*, 2020). Figure 2.2 is a diagrammatic illustration of hard tissues in the human body, with the knee being one of these hard tissues (Gobbi *et al*., 2020).

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For orthopaedic uses, like replacements of the knee, biomaterials are employed to manufacture prosthetic devices (Kumar *et al*., 2021). There are four cruciate ligaments in the knee joint, which function as a four-bar linkage system in a parasagittal plane (Kumar *et al*., 2021). The knee is comprised of three primary bone structures: the fibula, femur, and patella. The three main components of total knee replacements (TKRs) include the femoral, tibial, and patellar components (Kumar *et al*., 2021). The materials chosen for the femoral component of total knee replacement prostheses must meet specific criteria that may vary (Kumar *et al*., 2021).

Biocompatible materials, including polymers, ceramics, and metals, have been successfully used for biomedical applications. However, metals are preferred for load-bearing implants, such as knee implants, due to their unique strength. Titanium and its alloys are at the forefront of biomaterials research due to their exceptional biomechanical and biochemical attributes (Hon *et al*., 2003; Pietrocola *et al*., 2022).

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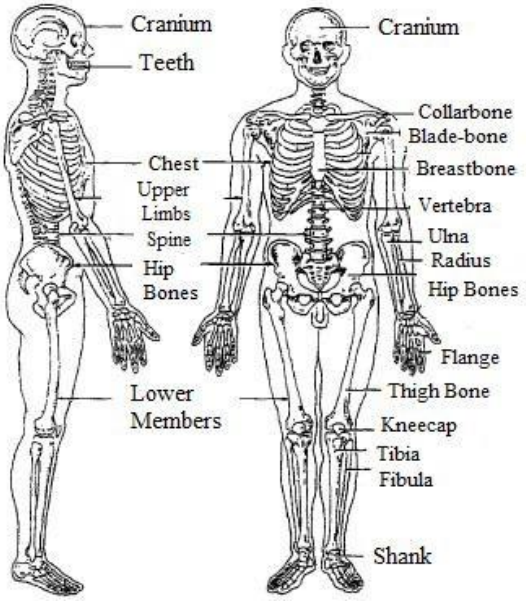


Figure 2.2: A diagrammatic representation of the hard tissues in the human body (Gobbi *et al*., 2020)

Titanium and its alloys are often referred to as 'wonder metals' due to their unique blend of biological, chemical, and physical characteristics, which make them indispensable in the production of biomedical devices. Titanium oxide forms a stable, thin, and protective film in oxidising environments through passivation or re-passivation, preventing corrosion and enhancing metal biocompatibility (Dzogewu and Du Preez, 2021). Ti-based alloys are chosen over other biomaterials for biomedical applications; however, it has been observed that the same surface characteristics that confer biocompatibility to Ti-based implants may also increase susceptibility to bacterial infections (Dzogewu and Du Preez, 2021; Geo *et al*., 2020).

Microbiological infections are a common problem associated with biomedical devices during both surgical and non-surgical procedures (Mikulskis *et al.*, 2018; Zamora-Mendoza *et al*., 2022). The frequent use of implants, surgical instruments, sutures, and catheters to treat various medical conditions exacerbates this issue (Zamora-Mendoza *et al*., 2022). Bacteria are the most prevalent microbes responsible for both acute and chronic infections, contributing to global morbidity (Khatoon *et al*., 2018; Zamora-Mendoza *et al*., 2022). Moreover, the increase in multidrug-resistant (MDR) bacteria driven by antibiotic overuse together with other elements that support

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bacterial growth, such as chronic colonisation in healthcare facilities and biofilm formation, has raised alarming infection rates (Wallace et al., 2020; Zamora-Mendoza *et al*., 2022). Common bacteria associated with biomedical devices include *Enterococcus faecalis*, *Proteus mirabilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus viridans*, and *Pseudomonas aeruginosa* (Zamora-Mendoza *et al*., 2022).

Under physiological conditions, a thin layer of titanium oxide develops on titanium surfaces, facilitating protein and cell adherence. However, this layer can also promote bacterial colonisation and biofilm formation on the Ti-based implant surface (Zamora-Mendoza *et al*., 2022). Therefore, without appropriate surface coatings, titanium and its alloys cannot fulfil therapeutic requirements, particularly regarding infection control (Dzogewu and Du Preez, 2021). Although antibiotics have been employed to coat implants, their effectiveness is diminishing due to rising antibiotic resistance. Consequently, this study aims to explore the use of essential oils for knee implant surface modification to combat infections caused by bacterial colonisation.

2.3. Background of Essential Oils

Essential oils are natural compounds derived from plants that exhibit antibacterial properties against a wide range of microorganisms (Burlec *et al.*, 2020; Aljaafari *et al*., 2020). They are typically extracted from plant materials using methods such as dry distillation, steam distillation, and other mechanical techniques that do not involve heating (Burlec *et al*., 2020). The chemical composition of essential oils can vary based on extraction methods, seasonal factors, and geographic location (Burlec *et al*., 2020). Essential oils can penetrate bacterial membranes and are effective against bacterial defences, such as biofilms (Setlhare, 2017). These oils prevent biofilm formation by disrupting quorum sensing and interfering with bacterial motility (Nuță *et al*., 2021). Numerous secondary metabolites found in essential oils possess the ability to inhibit or delay the growth of moulds, bacteria, and yeasts (Oulkheir *et al*., 2017; Ullah *et al*., 2022).

The essential oils of interest in this study, along with their primary components targeting knee-associated pathogens, are summarised in Table 2.1.

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Table 2.1: Advantages of *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils for health

Essential oil	Composition	Pathogens	Applications	References
Pelargonium *Asperum ssp* Egypte (geranium essential oil)	Geranio, geranic, citronellol, eugenol, linalool, citronellyl formate, citral, myrtenol, terpineol, methone, sabinene	*S. aureus*	*Pelargonium graveolens* oil has a very powerful antimicrobial action against *S. aureus* strains. *Pelargonium graveolens* oil has high alcohol compound content, possessing antibacterial qualities like geraniol and citronellol.	(Ali *et al*., 2015; Ibrahim and Kiki, 2020)
Melaleuca Alternifolia (tea tree essential oil)	Cymene, terpinene, *p*-, α-terpineol, α-terpinene , α-pinene, 1,8-cineole and terpinen-4-ol	*S. aureus, S. epidermidis*	*Melaleuca alternifolia* damages the membrane of the cell and prevents cellular respiration in *S. aureus*. *Melaleuca alternifolia* has anti-inflammatory effects due to its antifungal, antiseptic, and antibiotic properties.	(Cooney and Cooney, 2011; Ali *et al.,* 2015)

The extraction of essential oils employs various methods that can enhance their bioactive and therapeutic properties (Herman *et al*., 2019). Effective extraction techniques include gas chromatography tests, hydrolysis, freeze-drying, rotary evaporation, and steam distillation (Herman *et al*., 2019). As illustrated in Figure 2.3, steam distillation is the predominant method, responsible for extracting approximately 93% of essential oils, while the remaining 7% is obtained through alternative techniques (Herman *et al*., 2019). During the hydrolysis process, plant materials are immersed in water and subsequently boiled, allowing the surrounding water to protect the oils from scorching (Tongnuanchan and Benjakul, 2014; Herman *et al*., 2019).

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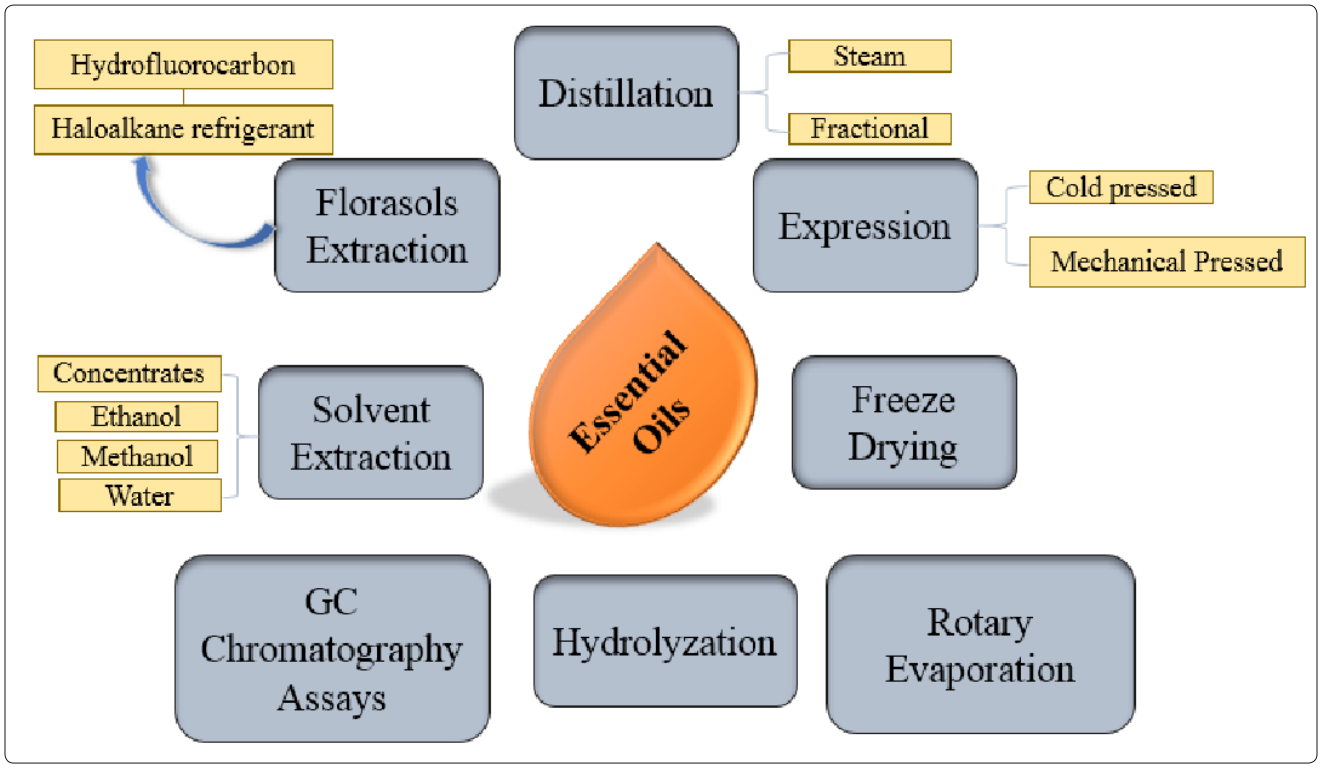


Figure 2.3: Various methods for the extraction of essential oils from diverse plant sources (Herman *et al*., 2019)

2.4. Antimicrobial Effects of Essential Oils Against Antibiotic-Resistant Bacteria

The prevalence of antibiotic-resistant bacterial strains, such as *S. aureus* and *S. epidermidis*, has increased, limiting the availability and raising the costs of antibiotics such as penicillin and methicillin for treating knee infections (Li and Webster, 2018; Ullah *et al*., 2022). Although antibiotics like penicillin are available to treat various bacterial pathogens, the severity of infections caused by these pathogens has escalated due to the growing resistance of multidrug-resistant bacteria to treatment (Swamy *et al.*, 2016; Ullah *et al*., 2022). In this regard, natural medicinal plants may offer potential antibacterial alternatives to traditional antibiotics (Ullah *et al*., 2022).

Essential oils have been documented to possess antifungal, insecticidal, and antiviral properties (Rosato *et al*., 2020). Literature suggests that the chemical composition of essential oils, including alcohols, phenols, aldehydes, esters, ketones, oxides, monoterpenes, and diterpenes, plays a

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significant role in their efficacy as antibacterial agents (Setlhare, 2017). The mechanisms of action of essential oils primarily involve the disruption of bacterial cell walls, damage to cell membranes through the formation of pores, and the leakage of cellular contents, as well as the impact on enzymes and membrane proteins (Jain *et al.*, 2018; Setlhare, 2017).

Essential oils are characterised by the presence of two or three primary components at higher concentrations (20-70%), distinguishing them from other substances found in trace amounts (Knezevic *et al*., 2016). Chemically, essential oils are composed of terpenes and their oxygenated derivatives, as well as aliphatic acids and phenolic and aromatic compounds, with composition varying among plant species and their respective parts (Knezevic *et al*., 2016). The hydrophobic nature of essential oils allows them to interact effectively with the lipids found in bacterial membranes (Knezevic *et al*., 2016).

Essential oils play a crucial role in protecting plants from herbivores by reducing herbivory and serving as antibacterial, antiviral, antifungal, and insecticidal agents (Knezevic *et al*., 2016). Certain essential oil constituents have been recognised as potential antibacterial agents, and the Department of Health and Human Services (HHS) has classified these oils as safe for use. Numerous studies have demonstrated the efficacy of essential oils against foodborne pathogens and infectious agents, suggesting their potential applications in the food industry (Knezevic *et al*., 2016).

Despite the incomplete understanding of their precise mechanisms of action, essential oils are regarded as effective antibacterial agents (Ullah *et al*., 2022). Their antibacterial efficacy may be significantly influenced by interactions with genetic material, disruption of enzyme pathways, or interactions with the phospholipid bilayer of bacterial cells (Ullah *et al*., 2022).

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2.5. Chapter Summary

The demand for knee replacement surgery is growing globally; however, the management of knee infections remains crucial due to their substantial threat to public health, particularly for vulnerable populations. Knee implantology has been developed to repair damaged knees, utilising implants to replace compromised joints. Nevertheless, implant failure may occur either early during the implantation process or later due to infection. Staphylococci are the predominant etiological agents implicated in knee infections, with bacterial colonisation around the implant leading to dense growth on its surface. Bacterial colonisation on the implant can result in severe consequences, including significant morbidity and financial burdens on healthcare systems.

Prophylactic systemic antibiotic regimens have been proposed as strategies to prevent infection during knee implant surgeries. However, the excessive use of antibiotics has led to the emergence of resistant microorganisms, highlighting the need for alternative treatments. Essential oils and other medicinal plants have demonstrated efficacy in preventing the emergence of drug-resistant bacterial strains that are challenging to treat with conventional antibiotics. In addition to their antibacterial properties, essential oils possess antioxidant and anti-diabetic effects, and are utilised in the treatment of cardiovascular diseases and cancer-related ailments. However, the impact of essential oils on the health and functionality of knee implants remains uncertain. This gap in knowledge drives ongoing research efforts to identify novel and potent antimicrobial agents derived from natural compounds, particularly those utilised in aromatherapy, traditional medicine, and cosmetic applications.

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CHAPTER THREE:
MATERIALS AND METHODS

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MATERIALS AND METHODS

3.1. Introduction

The emergence of antibiotic resistance in *S. epidermidis* and *S. aureus*, particularly in relation to knee implants, poses a significant challenge to global healthcare, necessitating the investigation of alternative therapeutic approaches beyond traditional antibiotics. Prior research (Setlhare, 2017; Ntondini *et al*., 2021) has established that essential oils are capable of inhibiting bacterial growth and compromising the structural integrity of bacterial membranes and cytoplasmic contents. In this study, *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils were selected for their documented efficacy against a wide range of bacteria, specifically targeting those associated with knee implant infections, namely *S. aureus* and *S. epidermidis* (Baker and Grant, 2018).

To ascertain the chemical composition of the selected essential oils, gas chromatography-mass spectrometry (GC-MS) was employed. Subsequently, the antibacterial activity of *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils against *S. epidermidis* and *S. aureus* was evaluated using a bioassay preparation method as recommended by Kock *et al*. (2000). The MIC was then determined to establish the specific concentrations at which these essential oils effectively inhibit the growth of both bacterial strains, as supported by relevant literature (Abidin *et al*., 2013; Desam *et al*., 2019). Building on the findings from SEM and TEM, which significantly demonstrated the antimicrobial effects of *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils against both *S. epidermidis* and *S. aureus* (Puvača *et al.*, 2019; Zazharskyi *et al*., 2020; Mohammed *et al.*, 2024), the next phase of the study focused on the efficacy of these essential oils against bacteria with Ti6Al4V experimental knee implants incorporated in the samples.

Ti6Al4V experimental knee implants were produced and the effective antioxidant properties of essential oils against bacteria were examined on the experimental knee implants' surface utilisig microdilution assay and SEM and TEM imaging to see how the essential oils alter the bacterial cells' structural makeup.

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3.2. Materials

3.2.1. Essential Oils Used in the Study

Essential oils derived from *Melaleuca alternifolia* (tea tree essential oil) and *Pelargonium graveolens* (geranium essential oil) were purchased from local suppliers.

3.2.2. Strains Used

Microorganisms were acquired from Laboratory Specialities (Pty) Ltd, South Africa. The tested strains were *S. aureus* (ATCC 25923) and *S. epidermidis* (ATCC 12228).

3.2.3. Materials for Manufacturing the Experimental Ti6Al4V Knee Samples

The experimental work was conducted using spherical Ti6Al4V powder, which was atomised in an argon atmosphere (see Figure 3.1), obtained from TLS Technik GmbH. Details on the powder's chemical makeup are provided in Table 3.1. Flowability testing recorded a Hausner ratio of 1.13 and a Carr index of 14.88, indicating a high likelihood of smooth powder delivery onto the powder bed (Ramosena *et al*., 2019).

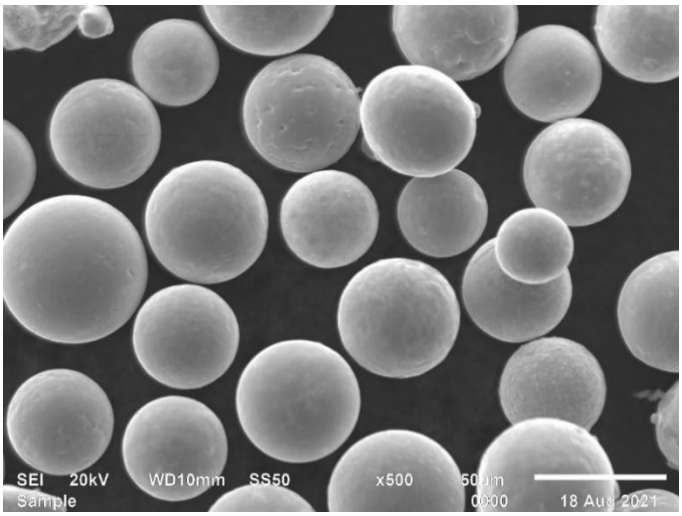


Figure 3.1: Spherical argon atomised Ti6Al4V powder

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Table 3.1: Elemental composition of the Ti6Al4V

Element	Ti	Al	V	O	N	H	Fe	C	Y
Content Wt.%	Bal	6.34	3.94	0.058	0.006	0.001	0.025	0.006	0.001

3.3. Methods

3.3.1. Essential Oil Characterisation

Essential oils derived from *Melaleuca alternifolia* and *Pelargonium graveolens* were acquired from local sources. These essential oils were classified based on the technique described by Kirbaslar *et al*. (2009). Hexane was used to briefly dissolve the oils (10% hexane) then administered a Finnigan Focus gas chromatograph (GC) at a split ratio of 50:1. The temperature of the injector was set at 230°C. An AB-1MS (30m x 0.25µm) capillary column was installed in the GC. The carrier gas utilized was helium, which flowed at a steady 1 mL min-1. The program's temperature was set at 240°C for four minutes before being elevated to 5˚C min-1 to 200˚C after which it was held at 200˚C for 1 minute and then elevated to 220°C, where it was kept for 10 min. The oils were subjected to mass analysis utilising 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 found by searching commercial libraries. After the identification procedure, MS data was expertly matched.

3.3.2. Bioassay Preparation

A method for quantitative microbiological bioassay was utilized to evaluate the antibacterial efficacy of essential oils on bacterial 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 0.1 ml was smeared on PCA (0.5% m.v-1 agar). This created a lawn that was uniform over the entire agar surface (Kock *et al*., 2009).). Then, a well (diameter and depth of 0.5 cm) was built in the centre of the Petri dish and 46 µl of essential oils together with ethanol were added The plates were all kept in the incubator at 37°C until regions of growth of different textures were noticeable (after 24

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h). To prevent essential oils from evaporating from the plates, the oils tested were diffused in the agar prior to incubation. After that the inhibition growth zones were detected and measured in diameters (mm).

3.3.3. Microdilution Assay

Microdilution essay was conducted, the bacterial strains' inoculum were prepared in Mueller-Hinton broth (MHB) for about 24 hours, and were modified to meet 0.5 McFarland turbidity standard (roughly 1.5 x 10^8 cfu/mL). Every suspension of bacteria was then dispersed into a 96-well sterile microtiter plate as displayed by Abidin *et al*. (2013) and Desam *et al*. (2019). The initial row of holes was filled with each essential oil and dilutions in series were carried out to achieve the desired concentrations of 12.5, 6.25, 3.125, 1.56, 0.8, 0.4, 0.2 and 0.1 µg/ml. The plate was closed by using sealer of sterile plate and then grown under controlled conditions in the laboratory at 37°C overnight. To demonstrate development of growth in a 24 hour period, the test was done two times for each concentration in duplicate. Following this, each well was then filled with p-iodonitrotetrazolium violet (INT) of about 20 µl. The plate was then put in the incubator at 37°C for 20 minutes. Growth was seen by a shift in colour from pink to violet.

3.3.4. Manufacturing of the Experimental Ti6Al4V Knee Samples

The Electro Optical System (EOS M280), a direct metal laser sintering (DMLS) machine which is part of laser powder bed fusion (LPBF) processes, was employed in the production of the samples of experimental implants. The direct metal laser sintering machine primarily comprises a process chamber equipped with a device for recoating, a computer-controlled elevating system, a heating module for a platform, an optical system which is equipped with a 400-watt fibre laser, a process gas management system, and a computer process with a control software (Chua *et al.*, 2010). The direct metal laser sintering technology for manufacturing belongs to a subset of additive manufacturing (AM), which is commonly referred to as 3D printing (Tao and Leu, 2016).

The direct metal laser sintering technology is regarded as a disruptive technology, marking the resurgence of the manufacturing sector (Dzogbewu, 2017a; Dzogbewu, 2020a). The technology is a manufacturing monolith with that has the unique ability to create intricate shapes (such as customized porous architectures, elaborate cooling channels, back tapers, and unique lattices or

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hollow structures) which could not be produced using traditional techniques (Yadroitsev *et al*., 2009). The direct metal laser sintering method uses a layer-by-layer production approach to generate 3D objects from the single tracks, which are set up using the computer-aided design (CAD) model (Dzogbewu *et al*., 2017b; Dzogbewu, 2020b). The fundamental manufacturing procedure entails a laser beam scanning the surface of a thin powder layer that has been applied to a substrate. Each laser scanning procedure is followed by the substrate being lowered up until the 3D object is manufactured in accordance to the CAD model. The manufacturing procedure is essentially an optimization method for eco-design topologies which enables the creation of very complex parts additively and monolithically (Dzogbewu et al., 2017), unlike traditional subtractive manufacturing techniques. The technology gives the engineer who designs freedom of design which is unchalleged and the capacity to create functional biomimetic implants with tailored, intricate, and architecture that is customised (Chua *et al*., 2010). The exceptional capacity for production of the direct metal laser sintering procedure, in contrast to traditional production techniques, allows for the production of biological items with specific mechanical characteristics for particular uses in biomedicine. The direct metal laser sintering has brought about the production of prosthetics with biomechanical characteristics similar to those of human bone, which has significantly increased the longevity of patients' implants (Gepreel and Niinomi, 2013). The powdered ingredients and substrate utilized in the production of the experimental knee samples had comparable chemical compositions. Argon served as the protective environment, additionally, the chamber's oxygen content was 0.07–0.1%. The ideal process parameters were used in the manufacturing of the samples: a 170 W laser power, scanning rate of 1.25 m/s, the hatch distance of 80 µm, and the thickness of the powdery layer of 30 µm with zigzag scanning strategy (Dzogbewu *et al*., 2016). Stress was reduced in the samples in an Ar atmosphere under 650°C conditions for three hours and were removed from the foundation plates by means of electrical discharge machining. To get rid of the powder particles that were loosely adhered, the samples were washed in an ultrasonic bath.

3.3.5. Microdilution Assay on Titanium Implant Materials

Due to the strong antibacterial properties of *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils, MICs were established using the titanium implant test samples. The inocula of the

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Strains of bacteria were created in Mueller-Hinton broth (MHB) for a period of 24 hours. Suspensions of the bacteria that were cultivated for 24 hours in Mueller-Hinton broth were modified to 0.5 McFarland turbidity standard (roughly 1.5 x 10^8 cfu/mL). Each suspension of bacteria was put into glass tubes with implant material inside of them (Abidin *et al.*, 2013). Essential oils of *Melaleuca alternifolia* and *Pelargonium graveolens* were added into the glass tubes' first row, and dilutions were carried out in series to reach the ultimate concentrations of 12.5, 6.25, 3.125, 1.56, 0.8, 0.4, 0.2 and 0.1 µg/ml. After that, the glass tubes were kept at 37°C for the whole night in a shaker incubator. For each concentration, the experiment was conducted twice in duplicate to show growth after 24 hours. After that, 20 µl of p-iodonitrotetrazolium violet (INT) was put into every glass tube. Next, for 20 minutes, the glass tubes were incubated at 37°C. The color changed from pink to violet, revealing growth.

3.3.6. Scanning Electron Microscopy (SEM) on the Titanium Implant Materials

SEM was employed to evaluate the resulting structural alterations due to the antibacterial action of *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils on *S. aureus* and *S. epidermids* on the titanium implant's surface. Getting cells ready for assessment using SEM was completed in accordance with the guidelines suggested by Ncango *et al*. (2010). Bacterial cells, both treated and untreated on the titanium implant material's surface (utilising the microdilution assay method) were primarily fixed utilising 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 about an hour. In order to dehydrate the titanium implant material, a succession of ethanol solutions was graded (30%, 50%, 70%, 90%, and 100% for 20 min for each solution, and the 100% dehydration was carried twice for 1 hour). After that, the ethanol-dehydrated titanium implant material was heated at 40°C and followed by sputter coating with uranium for 30 min to produce electrical conductivity. Next, the coated implant was inspected utilising SEM. To examine the structural alterations brought about by essential oils on the bacterial cells, pictures were obtained.

3.3.7. Transmission Electron Microscopy (TEM) on the Titanium Implant Materials

Transmission electron microscopy was utilised to evaluate the structural modifications that had happened because to the antibacterial action of *Melaleuca alternifolia* and *Pelargonium*

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graveolens essential oils on *S. aureus* and *S. epidermids* on the titanium implant material's surface. Getting the cells ready for assessment utilising transmission electron microscopy was completed in accordance with to the suggested procedures by van Wyk and Wingfield (1991). Bacterial cells, both treated and untreated on the titanium implant material's surface (utilising the microdilution assay procedure) were fixed primarily utilising 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 about an hour. In order to dehydrate the titanium implant material, a succession of ethanol solutions was graded (30%, 50%, 70%, 90%, and 100% for 20 min for every solution, and the 100% dehydration was carried twice for 1 hour). After that, the ethanol-dehydrated titanium implant material was heated at 40°C and followed by sputter coating with uranium for 30 min to produce electrical conductivity. Next, the coated implant was inspected utilising transmission electron microscopy. To examine the structural alterations brought about by essential oils on the bacterial cells, pictures were obtained.

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CHAPTER FOUR:
RESULTS AND DISCUSSION

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RESULTS AND DISCUSSION

4.1. Chemical Composition of the Selected Essential Oils

The chemical constituents of *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils are crucial to understanding their modes of action, particularly in the context of their antibacterial properties. Tables 4.1 and 4.2 present the detailed chemical profiles of these essential oils, as determined through gas chromatography-mass spectrometry (GC-MS). The analysis of *Melaleuca alternifolia* revealed the presence of 20 distinct compounds, with notable components including α-pinene (21.64%), γ-terpinene (21.09%), terpinen-4-ol (27.31%), limonene (12.3%), and cymene (10.1%), as highlighted in Table 4.1. In contrast, *Pelargonium graveolens* exhibited a more complex profile, identifying 27 different compounds. Dominant constituents included citronellol (42.2%), geraniol (21.7%), isomenthone (15.3%), citronellyl formate (14.7%), linalool (19.8%), geranyl formate (16.3%), and trans-Calamenene (13.2%) (see Table 4.2).

According to Badr *et al*. (2023), terpinen-4-ol, which constitutes a significant portion of *Melaleuca alternifolia* essential oil at 41.11%, possesses notable antibacterial properties that enable it to disrupt the morphological and functional integrity of bacterial membranes. The current study supports this assertion, identifying terpinen-4-ol as a key component of *Melaleuca alternifolia* essential oil at 27.31% (see Table 4.1), which directly correlates with its ability to compromise the membranes of *S. aureus* and *S. epidermidis*. Other notable constituents of *Melaleuca alternifolia* essential oil, including α-pinene, γ-terpinene, limonene, and cymene, have also been associated with antibacterial and anti-inflammatory properties (Kamel *et al*., 2022).

Fayoumi *et al*. (2022) conducted a GC-MS analysis of *Pelargonium graveolens* essential oil, identifying citronellol (30.5%) and geraniol (12.8%) as major components that contribute to cell wall disruption, loss of cellular contents, and overall bacterial deformation upon exposure to *Pelargonium graveolens* essential oil. The present study corroborates these findings, identifying similar major components in *Pelargonium graveolens* essential oil (see Table 4.2).

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Table 4.1: Chemical components of *Melaleuca alternifolia* essential oil

Number	Compound	Relative (%)
1	3-Thujene	0.7
2	β-Myrcene	1.1
3	β-Pinene	0.4
4	Cymene	10.1
5	terpinen-4-ol	27.31
6	α-Terpineol	2.2
7	Trans-Ascaridol glycol	3.2
8	1,4-dihydroxy-p-menth-2-ene	0.5
9	limonene dioxide	2.8
10	γ-terpinene	21.09
11	Ethanone,1-(6-methyl-7-oxabicyclo [4.1.0]hept-1-yl)-	1.0
12	Sobrerol	0.3
13	Globulol	1.9
14	α-pinene	21.64
15	Geranyl propionate	2.5
16	Geranyl valerate	3.0
17	γ-Eudesmol	3.4
18	Citronellyl tiglate	2.6
19	limonene	12.3
20	Geranyl tiglate	0.1

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Table 4.2: Chemical components of *Pelargonium graveolens* essential oil

Number	Compound	Essential oil (%)
1	Citronellol	42.2
2	Linalool	19.8
3	geranyl formate	16.3
4	α-Pinene	2.4
5	citronellyl formate	14.7
6	β-Pinene	3.4
7	Myrcene	0.1
8	Isomenthone	15.3
9	Limonene	1.9
10	Linalool	12.6
11	6,9-guaiadiene	2.3
12	Isomenthone	2.4
13	10-epi-γ-eudesmol	1.4
14	Isomenthol	1.1
15	α-Terpineo	1.0
16	Nerol	4.0
17	geranyl tiglate	0.7
18	Geranial	1.2
19	Neryl formate	1.3
20	2-phenyl ethyl tiglate	0.9
21	10-epi-γ-Eudesmol	1.1
22	Geranyl butyrate	1.4
23	Selina-4(15),7(11)-diene	1.6
24	Zonarene	0.9
25	δ-Cadinene	0.6
26	trans-Calamenene	13.2
27	Geraniol	21.7

Moreover, Galea *et al*. (2023) highlighted that the presence of citronellol and geraniol in *Pelargonium graveolens* essential oil endows it with antifungal, anti-inflammatory, and antimicrobial attributes. The ability of *Pelargonium graveolens* essential oil to reduce

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inflammation can alleviate joint pain and enhance mobility in affected areas. This study further confirms the presence of both citronellol and geraniol in *Pelargonium graveolens* essential oil, which may explain its effectiveness in the inhibition growth of *S. aureus* and *S. epidermidis* (see Table 4.2). Notably, over 40% of the components in *Pelargonium graveolens* essential oil consist of alcohol-based compounds that exhibit antibacterial characteristics, as documented by Bigos *et al*. (2012). *Pelargonium graveolens* essential oil is effective against strains of *S. aureus* and *S. epidermidis* with various drug-resistant mechanisms, making it a viable candidate for inclusion in the treatment of knee infections.

The use of essential oils like as *Melaleuca alternifolia* and *Pelargonium graveolens* in addressing human infections produced by bacterial strains that are resistant to many drugs presents an innovative alternative to synthetic pharmaceuticals. However, it is crucial to recognise that the percentage compositions of the identified constituents in essential oils may differ significantly because of elements like as plant growth conditions, genetic variability, chemical forms, harvesting seasons, and nutritional status of the plants.

4.2. Antimicrobial Properties of Essential Oils

This study employed the bioassay method to evaluate the antibacterial properties of *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils against antibiotic-resistant strains of *S. aureus* and *S. epidermidis*. The bioassay was designed to identify essential oils exhibiting diameters of inhibition of at least 20 mm. Following this, the essential oils were applied to the surfaces of titanium knee implants to assess their efficacy in inhibiting bacterial growth. Both *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils demonstrated significant growth-inhibiting effects on *S. aureus* and *S. epidermidis*. Further investigations were conducted to determine the MICs of these essential oils and to examine the structural changes they induced in bacterial cells. The findings of this study indicate that *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils may be effectively utilised on the surfaces of titanium knee implants to stop the colonization of bacteria. However, further research is needed regarding essential oils' adherence to titanium knee implants, to be precise.

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4.2.1. Initial Key Findings

The inhibitory zones' findings are displayed in Table 4.3 and illustrated in Figure 4.1. The essential oils exhibited varying degrees of efficacy, which may be attributed to the specific modes of action of each oil against the bacterial species examined. As noted by Badr *et al*. (2023), terpinen-4-ol, a key component of *Melaleuca alternifolia* essential oil, along with citronellol and geraniol found in *Pelargonium graveolens* essential oil, may be responsible for the observed antimicrobial activity against *S. aureus* and *S. epidermidis* (Yasin *et al*., 2021; Galea *et al*., 2023; Badr *et al*., 2023).

Melaleuca alternifolia essential oil is known for its wide range of activity, which includes antifungal, antiviral, and antiprotozoal properties (Badr *et al*., 2023). The components of *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils demonstrated inhibitory effects, which likely contribute to their capacity to disrupt the morphological and the ability of bacterial membranes to function (Badr *et al*., 2023). This study confirmed that both *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils effectively inhibit the growth of *S. aureus* and *S. epidermidis*, as shown in Table 4.3 and Figure 4.1.

Table 4.3: Inhibition zone diameters of essential oils against pathogens

Essential oils	**Inhibition zone diameters of bacteria (mm)**	
	S. epidermis	*S. aureus*
***Pelargonium graveolens* (Geranium)**	24.6	32.8
***Melaleuca alternifolia* (Tea tree)**	20,5	39.1

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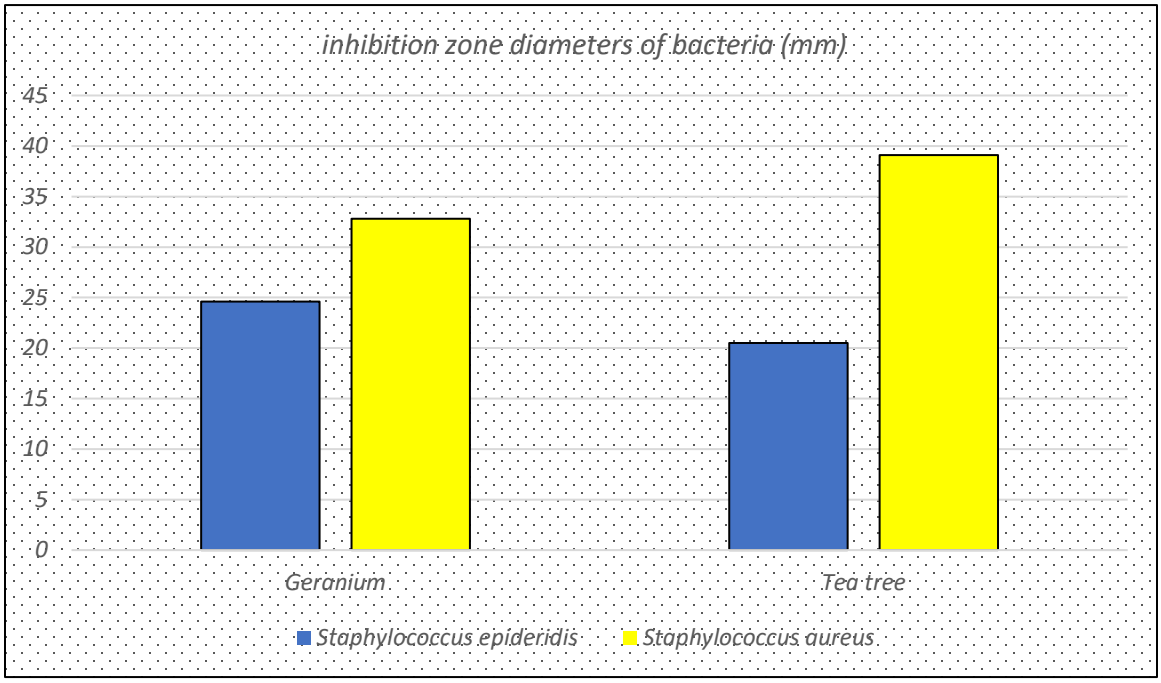


Figure 4.1: Graph illustrating the inhibition zone diameters of the tested essential oils against *S. epidermidis* and *S. aureus*

Specifically, *melaleuca alternifolia* essential oil exhibited the most substantial inhibitory effect on *S. aureus*, with the largest inhibition zone measuring 39.1 mm (see Table 4.3 and Figure 4.1). *Pelargonium graveolens* essential oil also demonstrated significant antimicrobial activity against *S. aureus*, with inhibition zones of 32.8 mm. Furthermore, *Pelargonium graveolens* essential oil effectively inhibited the growth of *S. epidermidis*, exhibiting an inhibition zone of 24.6 mm, while *Melaleuca alternifolia* essential oil showed an inhibition zone of 20.5 mm against the same strain (see Table 4.3 and Figure 4.1). Notably, a study by Abdelhamed *et al*. (2022) corroborated these findings, confirming that *Melaleuca Alternifolia* essential oil can prevent the growth of *S. epidermidis* (Abdelhamed *et al*., 2022).

Overall, these results underscore the potential of *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils as effective antimicrobial agents, highlighting their applicability in preventing infections associated with knee implants.

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4.2.2. Further Testing and Results

The findings from the bioassay preparation revealed that both essential oils exhibited significant antimicrobial activity, highlighting their potential for use as coatings on titanium knee implants to assess their efficacy in inhibiting bacterial growth on the implant surfaces. In this phase of the study, both *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils were selected for application on the titanium knee implants to stop the growth of *S. aureus* and *S. epidermidis*. However, before these oils were utilised on the implant surfaces, their MICs against the bacterial strains were determined through a microdilution assay.

Notably, each essential oil demonstrated varying responses against the different bacterial strains tested, a phenomenon that can be attributed to the distinct chemical compositions inherent to each oil. This variability emphasises the necessity of considering the specific interactions between essential oil constituents and bacterial species when developing antimicrobial coatings for biomedical applications.

To further evaluate the concentrations at which *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils inhibit the growth of *S. aureus* and *S. epidermidis*, a microdilution assay was conducted. The results indicated that both essential oils exhibited strong antibacterial activity. Specifically, the MIC value for *Pelargonium graveolens* essential oil against *S. aureus* was determined to be 0.40 µg/ml (see Table 4.4), which aligns closely with findings reported by Mahboubi *et al*. (2016), who noted that *Pelargonium graveolens* essential oil demonstrated potent activity against *S. aureus* isolates and multidrug-resistant strains, with MIC values ranging from 0.25 to 2.50 µg/ml. Similarly, Hsouna and Hamdi (2012) recorded MIC values of 0.312 and 0.625 µg/ml for *Pelargonium graveolens* essential oil against *S. aureus*.

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Table 4.4: Dilution of *Melaleuca alternifolia* essential oil and *Pelargonium graveolens* essential oil on *S. aureus*

***Melaleuca alternifolia* and *Pelargonium graveolens* essential oils on *S. aureus* and control (µg/ml)**	**≥12.5**	**≥6.25**	**≥3.13**	**≥1.6**	**≥0.8**	**≥0.4**	**≥0.2**	**≥0.1**
Melaleuca alternifolia	-	-	-	-	-	-	++	+++
Pelargonium graveolens	-	-	-	-	-	-	++	+++
Control	+++	+++	+++	+++	+++	+++	+++	+++

Table 4.5: Dilution of *Melaleuca alternifolia* essential oil and *Pelargonium graveolens* essential oil on *S. epidermidis*

***Melaleuca alternifolia* and *Pelargonium graveolens* essential oils on *S. epidermis* and control (µg/ml)**	**≥12.5**	**≥6.25**	**≥3.13**	**≥1.6**	**≥0.8**	**≥0.4**	**≥0.2**	**≥0.1**
Pelargonium graveolens	-	-	-	-	-	-	-	++
Melaleuca alternifolia	-	-	-	-	-	-	+++	+++
Control	+++	+++	+++	+++	+++	+++	+++	+++

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Additionally, *Melaleuca alternifolia* essential oil exhibited antibacterial effects against *S. aureus* with an MIC value of 0.40 µg/ml. Furthermore, *Pelargonium graveolens* essential oil showed an MIC value of 0.2 µg/ml and *Melaleuca alternifolia* 0.4 µg/ml against *S. epidermidis*. These MIC results collectively indicate that both *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils possess potent antibacterial activity against both *S. aureus* and *S. epidermidis*, as summarised in Tables 4.4 and 4.5, highlighting their relevance in the context of knee implants.

Elghali *et al*. (2024) suggested that the antibacterial effects of *Pelargonium graveolens* essential oil may be attributed to its chemical composition and the concentration of active molecules present. The robust antibacterial action of *Pelargonium graveolens* is likely linked to the abundance of geraniol and citronellol (Elghali *et al*., 2024). *Melaleuca alternifolia* oil, containing components such as α-pinene, γ-terpinene, terpinen-4-ol, limonene, and cymene, which were also identified in this study (see Table 4.2), has been individually associated with antibacterial and anti-inflammatory properties (Kamel *et al*., 2022).

4.3. Antimicrobial Action of Essential Oils on Titanium Knee Implants

Building on the findings presented in Section 4.2.2, further analysis was warranted to investigate the antimicrobial potential of *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils against bacterial colonisation on titanium knee implants. These essential oils have previously been utilised to address bacterial infections that colonise the surfaces of titanium implant materials, which can lead to early implant failure. It is well-documented that applying antibiotics, antiseptics, or other antimicrobial treatments to implant materials can effectively stop the development of bacteria on their surfaces (Khatoon *et al*., 2018; Woo *et al*., 2020). As such, the current research aimed to explore the application of essential oils as a strategy to prevent bacterial colonisation on titanium knee implants.

The antimicrobial effect of *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils on titanium knee implant materials was assessed qualitatively by determining the MICs of these oils against *S. aureus* and *S. epidermidis*. The microdilution method was employed for this purpose. Titanium implant materials were immersed in solutions containing the respective essential oils to evaluate their MICs. The results indicated that both *Melaleuca alternifolia* and *Pelargonium*

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graveolens essential oil solutions exhibited an MIC of 6.25 µg/ml against *S. aureus*, while a lower MIC of 3.13 µg/ml was observed for both oils against *S. epidermidis* (see Tables 4.6 and 4.7).

These essential oils were subsequently applied at their determined MICs to investigate the morphological changes they induced both externally and internally in bacterial cells. SEM and TEM were utilised to conduct a thorough examination of the structural alterations imposed by the essential oils on the isolates of *S. aureus* and *S. epidermidis*.

Table 4.6: Dilution of *Melaleuca alternifolia* essential oil and *Pelargonium graveolens* essential oil on *S. aureus*

***Melaleuca alternifolia* and *Pelargonium graveolens* essential oils on *S. aureus* and control (µg/ml)**	**≥12.5**	**≥6.25**	**≥3.13**	**≥1.6**	**≥0.8**	**≥0.4**	**≥0.2**	**≥0.1**
Melaleuca alternifolia	-	-	+	++	+++	+++	+++	+++
Pelargonium graveolens	-	-	+	++	+++	+++	+++	+++
Control	+++	+++	+++	+++	+++	+++	+++	+++

Table 4.7: Dilution of *Melaleuca alternifolia* essential oil and *Pelargonium graveolens* essential oil on *S. epidermidis*

Melaleuca alternifolia* and *Pelargonium graveolens* essential oils on *S.	**≥12.5**	**≥6.25**	**≥3.13**	**≥1.6**	**≥0.8**	**≥0.4**	**≥0.2**	**≥0.1**

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***epidermis* and control (µg/ml)**								
Pelargonium graveolens	-	-	-	+	++	+++	+++	+++
Melaleuca alternifolia	+++	+++	-	+++	+++	+++	+++	+++
Ethanol	+++	+++	+++	+++	+++	+++	+++	+++

Collectively, these findings affirm the potential of *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils as antimicrobial agents that are effective to prevent bacterial colonisation on titanium knee implants. The use of SEM and TEM allows for a comprehensive analysis of the morphological changes in bacterial cells, providing valuable insights into the mechanisms through which these essential oils work to combat bacteria. This innovative approach not only enhances our understanding of the interactions between essential oils and bacterial pathogens but also opens the path for the development of improved strategies for mitigating implant-associated infections.

4.4 Morphological Changes of Bacterial Cells in the Presence of Essential Oils

The rising prevalence of knee implants has led to a public health concern, particularly because of the rise in rates of antibiotic resistance (Malizos and Kirketerp-Møller, 2016). For instance, *S. aureus* has developed resistance to both methicillin and penicillin, rendering these antibiotics ineffective by limiting the formation of cell wall openings necessary for their action. While antibiotic molecules typically enter Gram-positive bacteria through diffusion via external membrane porins, decrease in the quantity of porin channels impedes antibiotic entry into the cell (Magunga, 2016; Setlhare, 2017; Ntondini *et al*., 2021). Furthermore, bacteria possess efflux pumps that actively expel antibiotics, including groups such as Tetracyclines, Lincosamides, Phenicols, and Lipopeptides (Ntondini *et al*., 2021).

These efflux pumps operate at a rate commensurate with the influx of antibiotics, effectively expelling them before they can reach their intracellular targets (Setlhare, 2017; Ntondini *et al*.,

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2021). The exterior membranes of Gram-positive bacteria act as barriers, preventing antibiotics from penetrating bacterial cells during their evolutionary adaptations. Consequently, microorganisms have developed resistance mechanisms that enable them to endure and grow in the midst of antibiotics, thereby limiting the effectiveness of antimicrobial agents. In contrast, essential oils have demonstrated the the capacity to stop the expansion of many bacterial and fungal pathogens (Setlhare, 2017; Ntondini *et al*., 2021). Essential oils can penetrate the bacterial cell wall, target the cytoplasm and membrane, and induce structural changes that inhibit pathogen growth (Setlhare, 2017; Ntondini *et al*., 2021).

To assess the effects of *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils on *S. aureus* and *S. epidermidis*, SEM and TEM techniques were employed. The SEM results revealed observable morphological changes, including slight roughness (R) of the cell surfaces, loss cellular content (LCC), and damage to the cell walls (DCW) (see Figures 4.2 and 4.5). These figures illustrate that the application of *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils adversely affected the cell wall structures of both treated *S. aureus* and *S. epidermidis*. The figures confirm a significant impact of the essential oils on the structural integrity of these antibiotic-resistant bacteria, as evidenced by the control cells, which exhibited no such changes (see Figures 4.2 and 4.5).

Furthermore, Kamel *et al*. (2022) have previously demonstrated the efficacy of *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils in inhibiting the growth of *S. aureus*, which is commonly associated with knee implant infections. This study further corroborates the effect of essential oils in combating bacterial infections in the knee. Additionally, prior investigations by Nguyen *et al*. (2023) indicated that *Melaleuca alternifolia* essential oil disrupts bacterial membranes and acts as a membrane permeabiliser, compromising the bacteria's ability to regulate their chemiosmotic processes for both Gram-positive and Gram-negative organisms (Kamel *et al*., 2022). Nguyen *et al*. (2023) also reported that *Melaleuca alternifolia* essential oil caused cell membrane disruption in *S. epidermidis*, leading to cell death. Correspondingly, this study also demonstrated the membrane disruption of both *S. aureus* and *S. epidermidis* by *Melaleuca alternifolia* essential oil (see Figure 4.2).

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The TEM results (see Figures 4.6 to 4.9) revealed drastic alterations in the cellular structure of *S. aureus* and *S. epidermidis* following exposure to *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils. Notable changes included damaged cell walls, the formation of holes, and the reduction in the contents of the cell, which leads to cell death. The observed intercellular leakage and morphological alterations in the treated cells confirm that *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils significantly impact the structural integrity of the cell walls of *S. aureus* and *S. epidermidis*. Thus, it can be argued that these essential oils possess the capacity to function as strong antibacterial against strains of *S. aureus* and *S. epidermidis* associated with knee implants.

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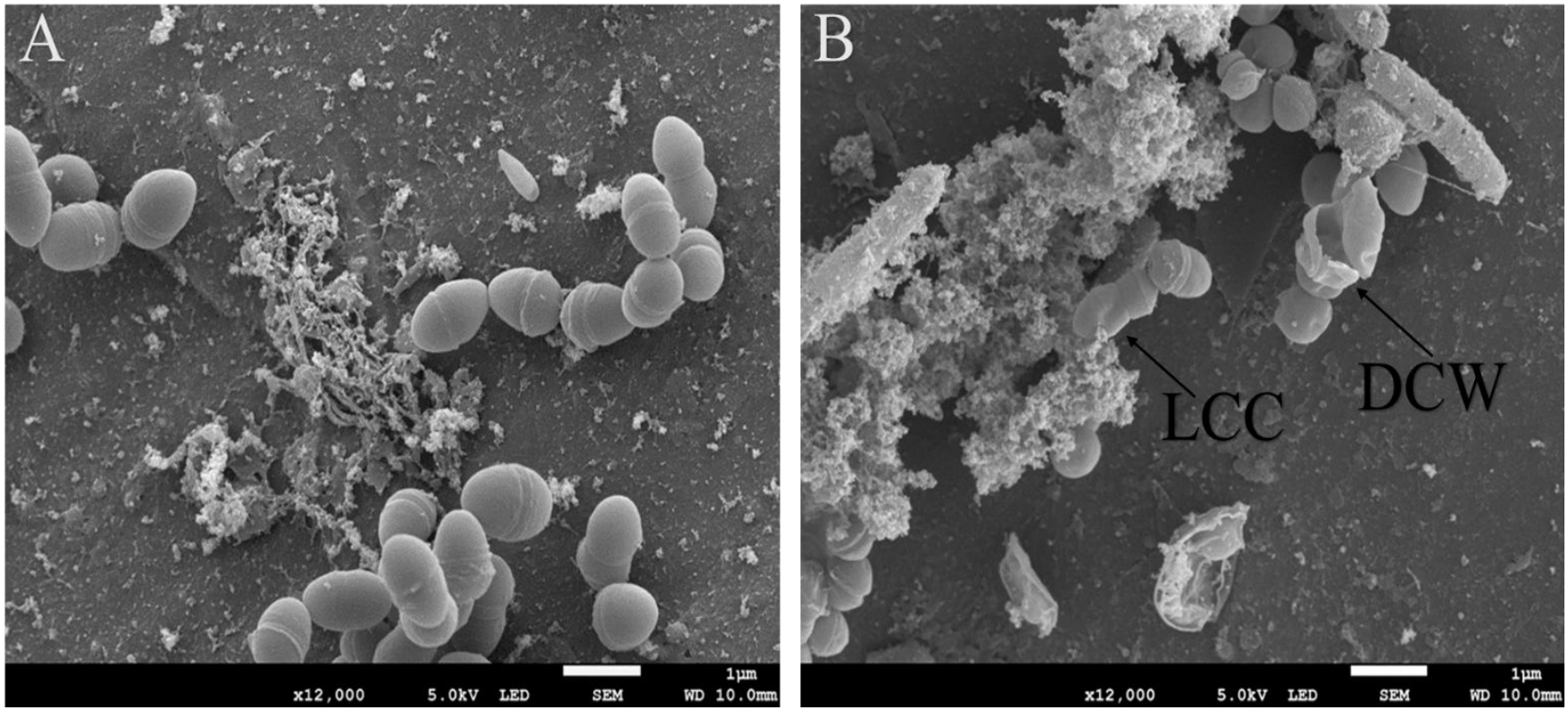


Figure 4.2: SEM picture A of a titanium implant presenting *S. epidermidis* cells in the control. Picture B shows *S. epidermidis* cells treated with *Melaleuca alternifolia* essential oil. Demonstrating a loss in cell content (LCC) and a damaged cell wall (DCW).

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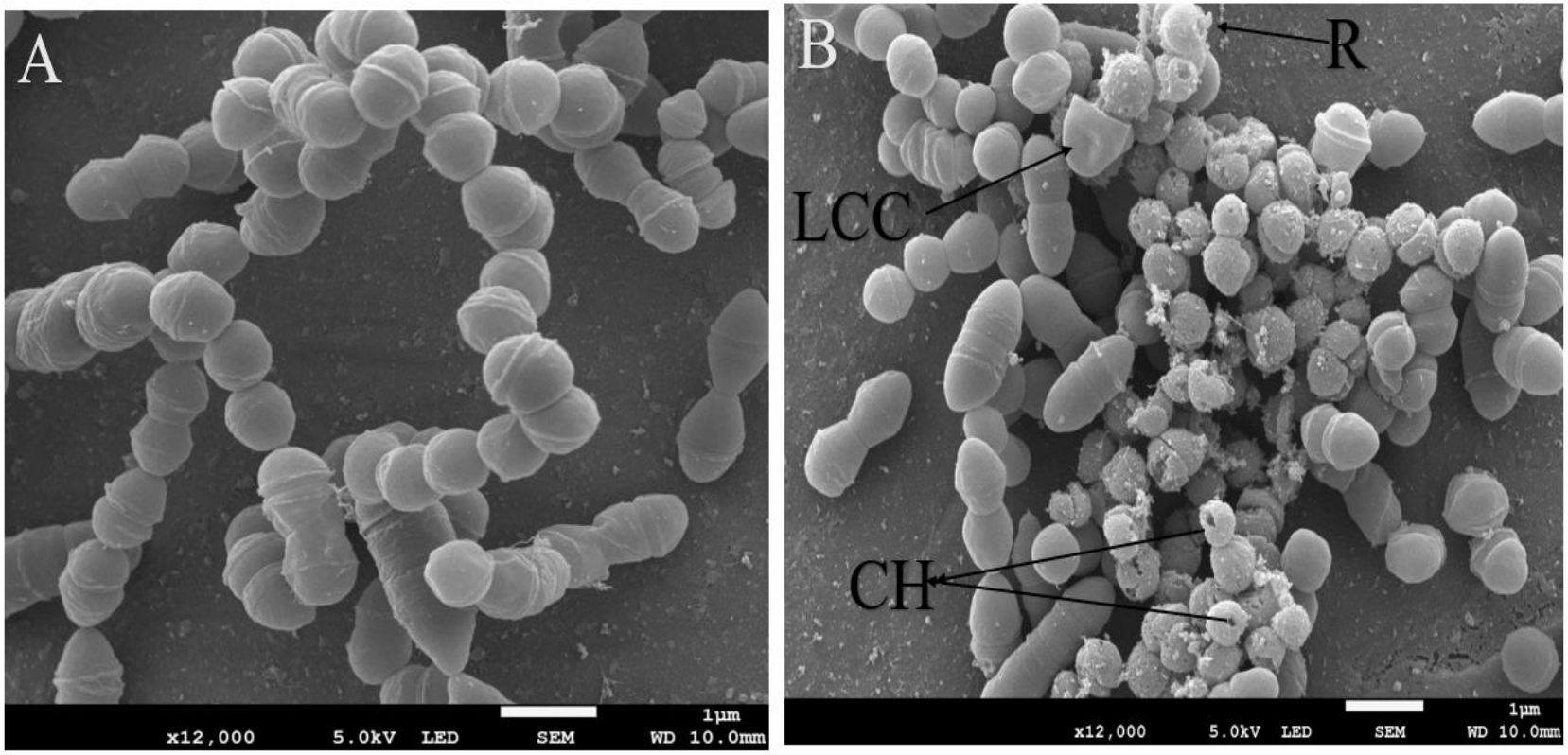


Figure 4.3: SEM picture A of a titanium implant presenting *S. aureus* cells in the control. Picture B presents *S. aureus* cells, which are treated with *Melaleuca alternifolia* essential oil. Demonstrating a loss in the content of bacterial cell (LCC), damaged cell wall (DCW), cell holes (CH), and roughness (R).

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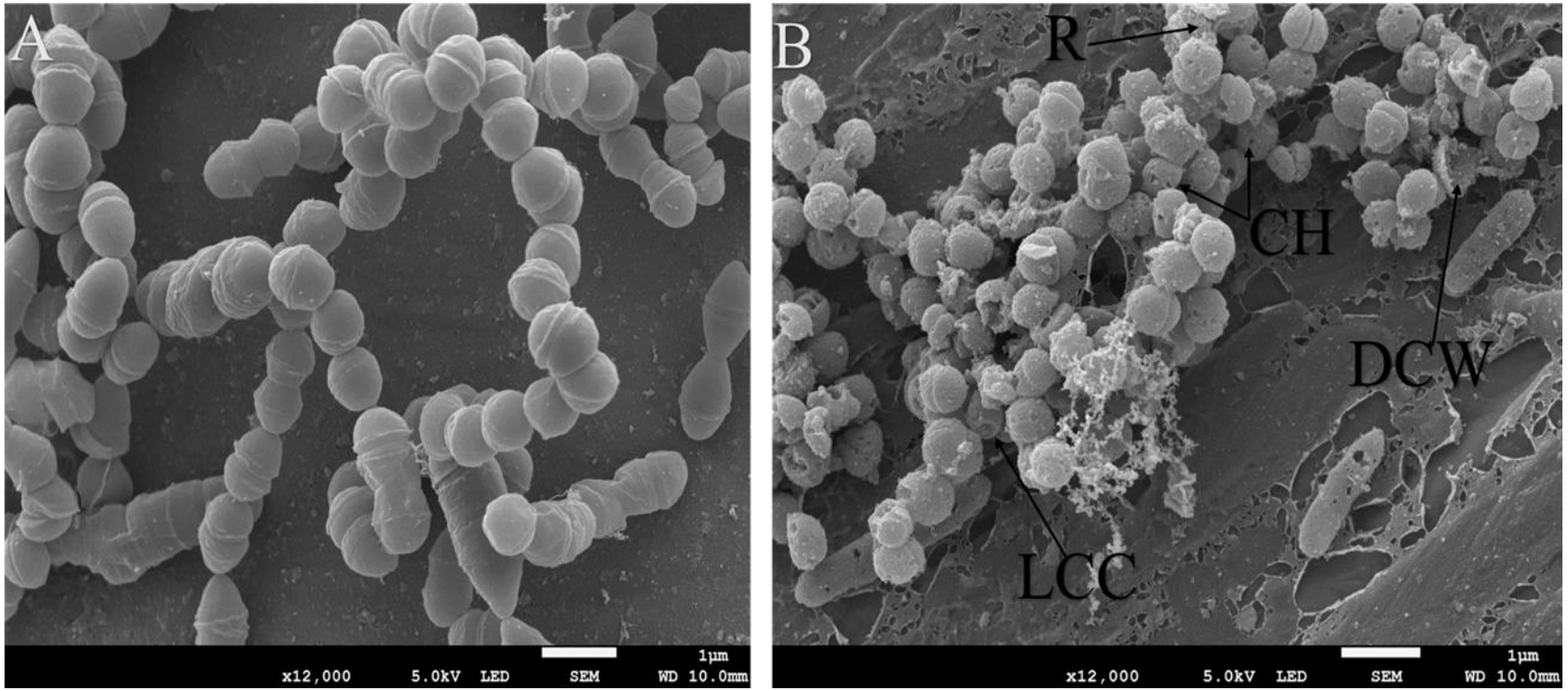


Figure 4.4: SEM picture A of a titanium implant presenting *S. aureus* cells in the control. Picture B presents *S. aureus* cells which are treated with *Pelargonium graveolens* essential oil. Demonstrating a loss in the content of bacterial cell (LCC), a damaged cell wall (DCW), cell holes (CH), and roughness (R).

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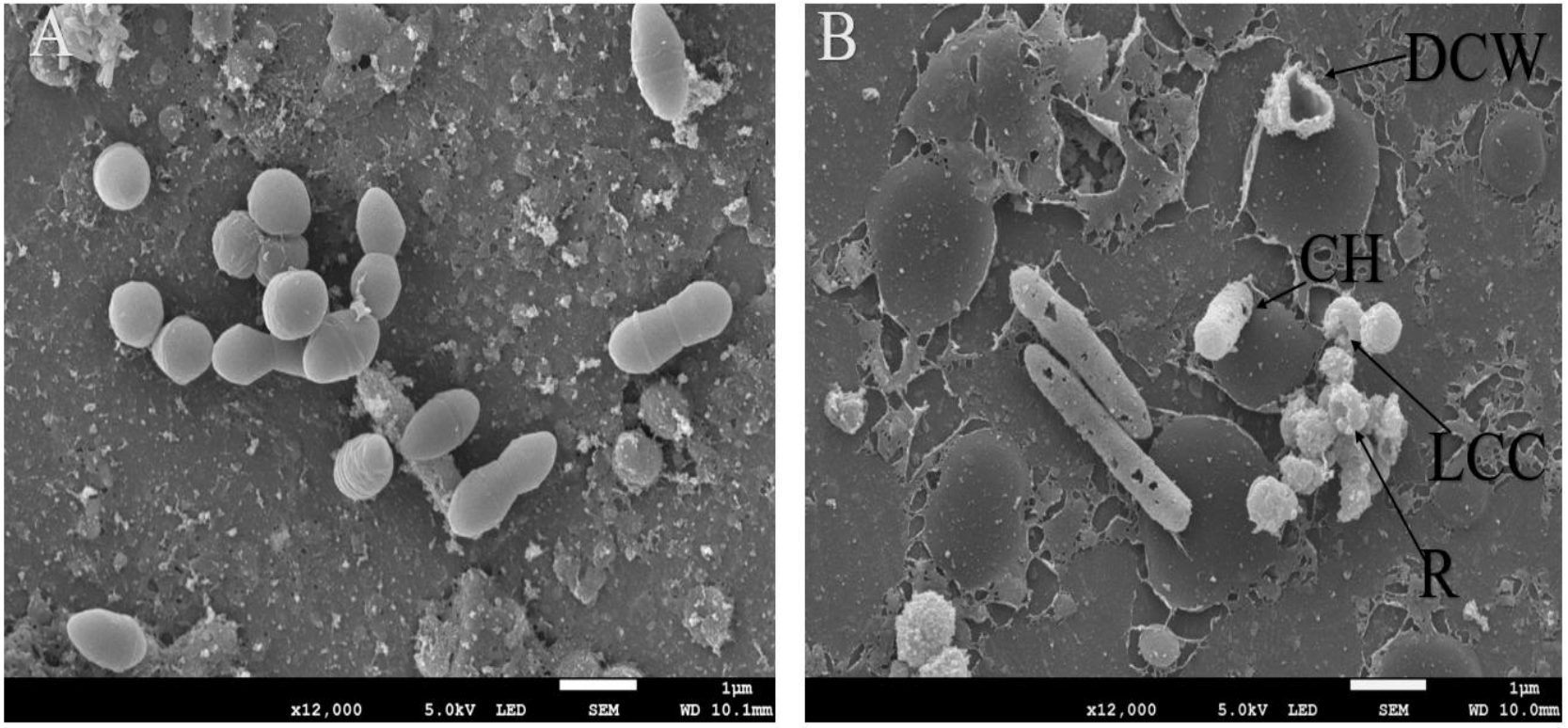


Figure 4.5: SEM picture A of a titanium implant presenting *S. epidermidis* cells in the control. Picture B shows *S. epidermidis* cells treated with *Pelargonium graveolens* essential oil. Demonstrating a loss in the content of bacterial cell (LCC), a damaged cell wall (DCW), cell holes (CH), and roughness (R).

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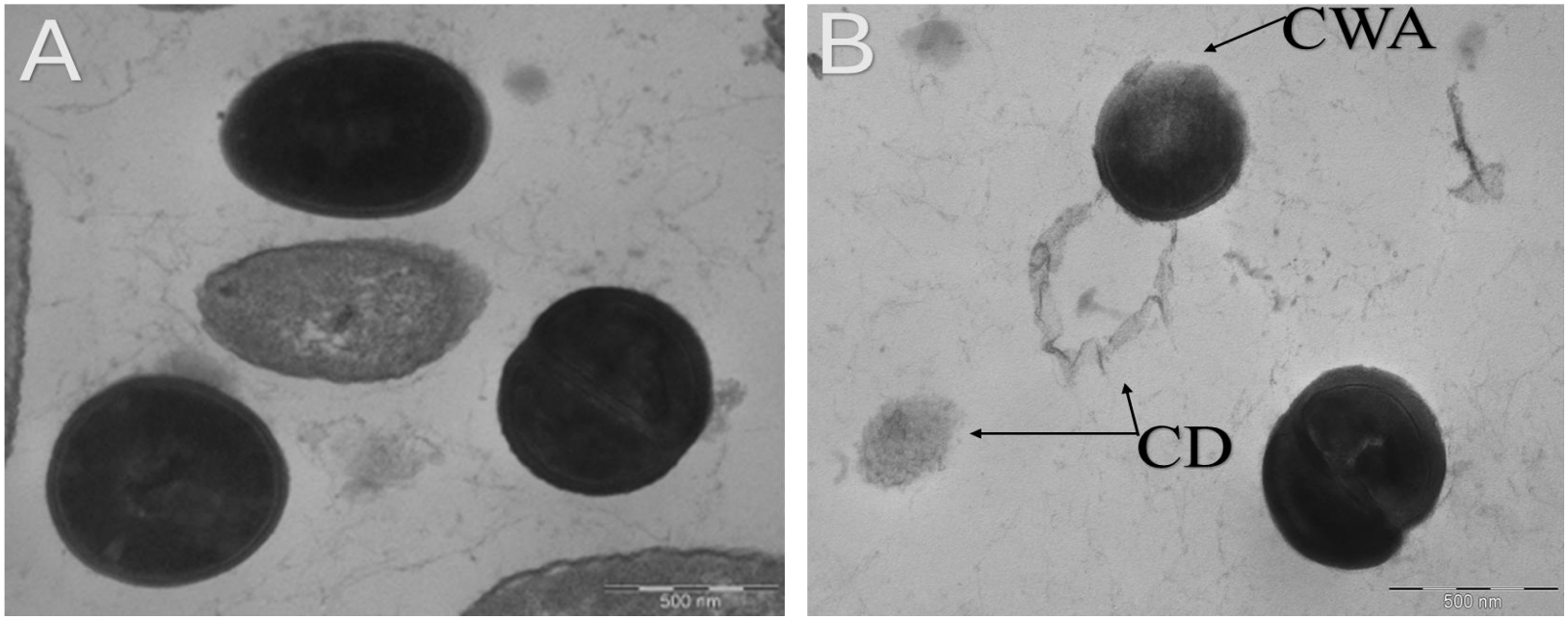


Figure 4.6: TEM picture A of a titanium implant representing *S. aureus* cell's control. Picture B represents the morphological changes of *S. aureus* cells, after being exposed to *Pelargonium graveolens* essential oil, with cellular debris (CD), and with the cell wall absent (CWA).

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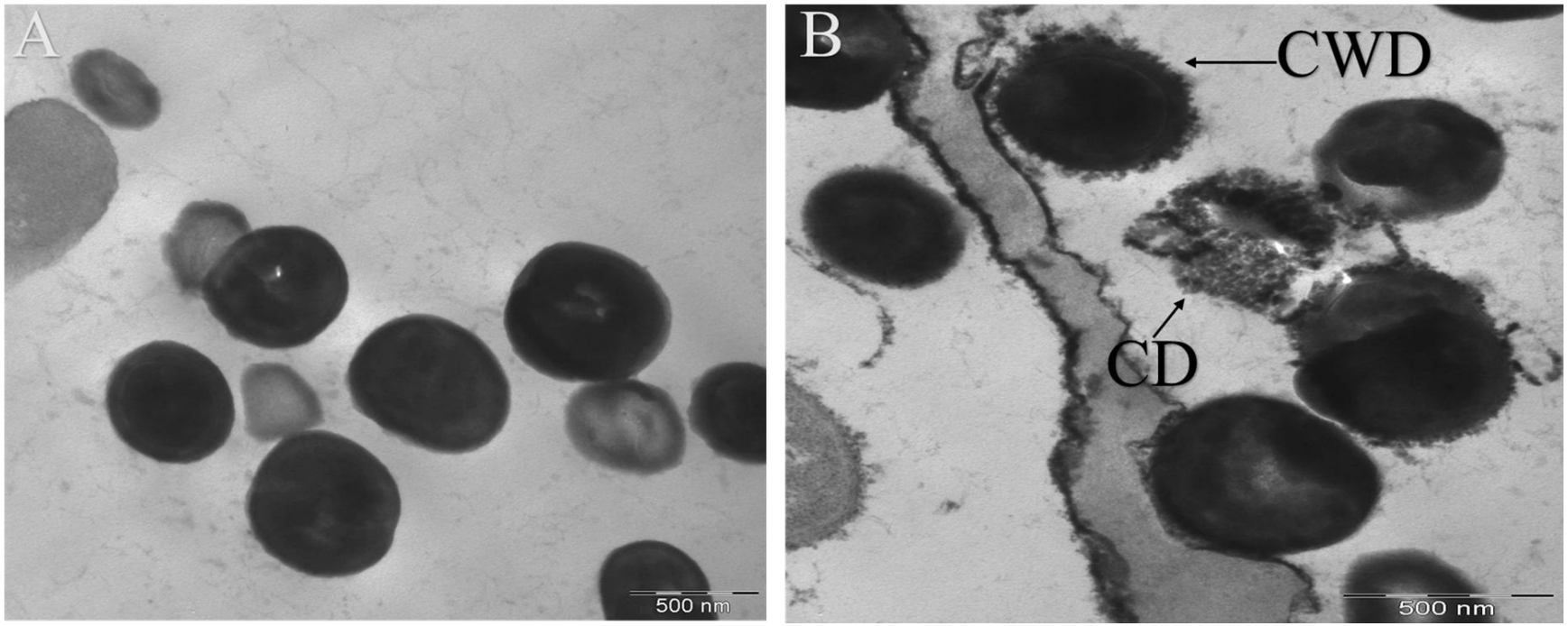


Figure 4.7: TEM picture A of a titanium implant) representing *S. epidermidis* cell's control. Picture B represents the morphological changes of *S. epidermidis* cells after being exposed to *Pelargonium graveolens* essential oil, with cellular debris (CD), and with the cell wall disintegrating (CWD).

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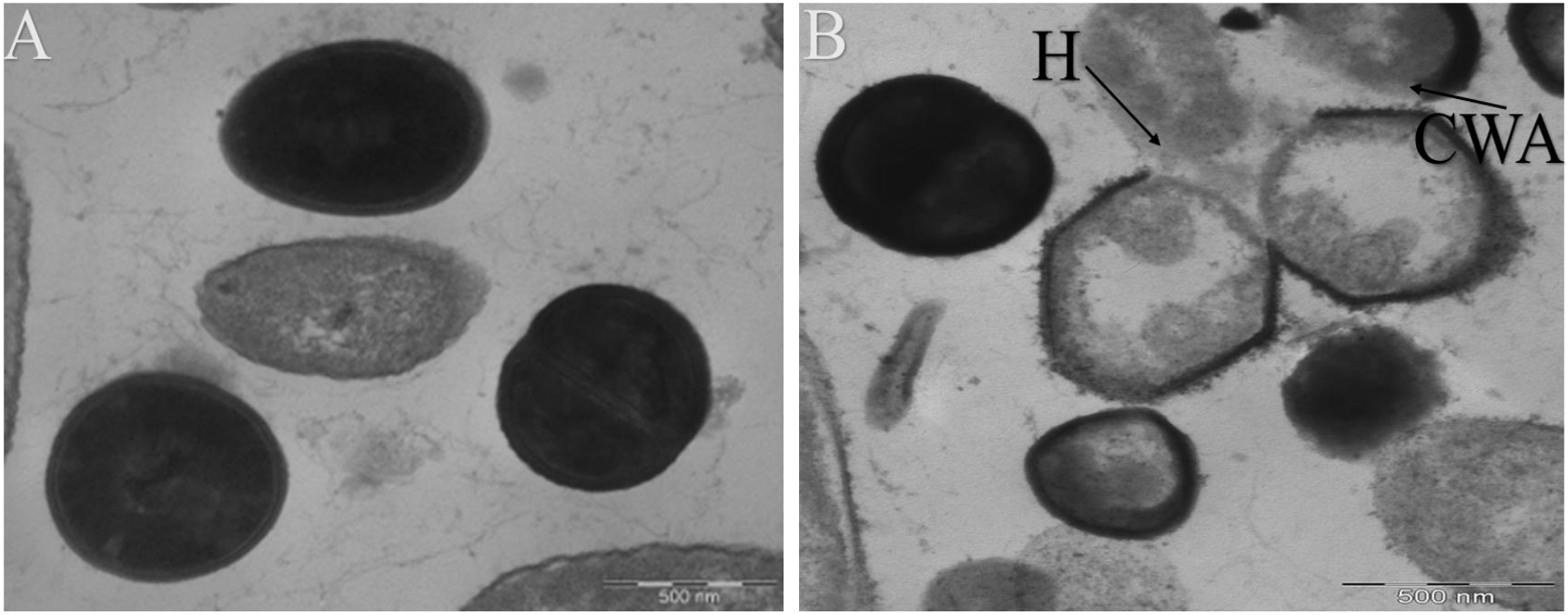


Figure 4.8: TEM picture A of a titanium implant representing *S. aureus* cells. Picture B represents the morphological changes of *S. aureus* cells after being exposed to *Melaleuca alternifolia* essential oil, with holes (H), and with the cell wall absent (CWA).

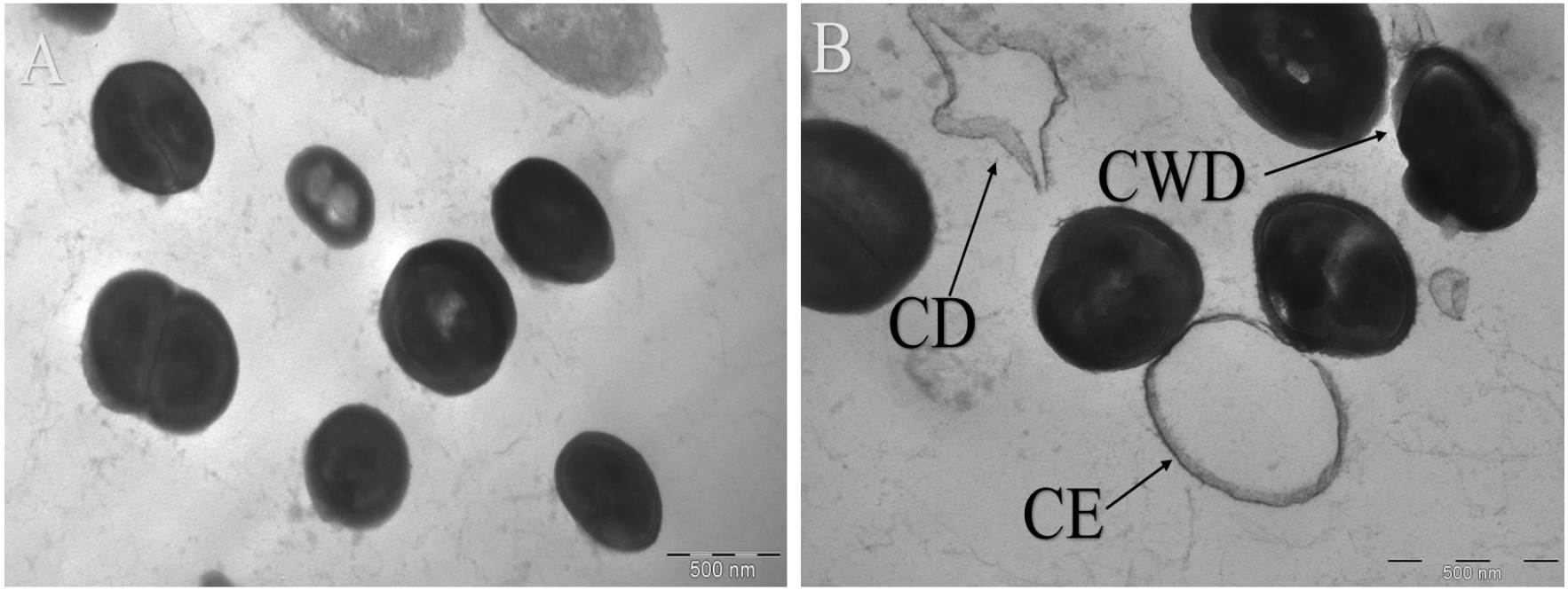


Figure 4.9: TEM picture of a titanium implant representing *S. epidermidis* cells' control. Picture B represents the morphological changes of *S. epidermidis* cells after being exposed to *Melaleuca alternifolia* essential oil, with cellular debris (CD), and with the cell wall disintegrating (CWD), and with the cell empty (CE).

4.5 Chapter Summary

In recent decades, biomaterials such as titanium have been developed to replace damaged bones, significantly enhancing regenerative healthcare. However, the increased risk of pathogen-induced infections presents a crucial challenge that presents a significant risk to both the economy and public health. The global prevalence of antibiotic-resistant bacteria has prompted researchers to seek new antibacterial solutions. Natural compounds, particularly essential oils, have emerged as promising alternatives against antibiotic-resistant bacteria. Consequently, this research aimed to investigate the antimicrobial activities of *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils against antibiotic-resistant bacteria associated with knee implants.

The findings of this research demonstrated that both *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils effectively inhibited the growth of *S. aureus* and *S. epidermidis,* that are frequently associated with knee implant infections. The bioassay results revealed significant antimicrobial activity, confirming the possibilities of these essential oils as potent antimicrobial agents against both bacterial strains. Additionally, the MIC results indicated that *Pelargonium graveolens* and *melaleuca alternifolia* essential oils could significantly reduce the growth of *S. epidermidis* and *S. aureus*, suggesting their applicability in the coating of knee implants to prevent infections.

As natural products, the essential oils utilised in this study exhibit strong antibacterial properties through their mechanisms of action, which include causing damage to the cytoplasmic membrane, breaking down the bacterial cell wall, and inducing cytoplasmic coagulation upon contact with bacterial cells. This research not only confirmed the potent antimicrobial properties of *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils but also proposed potential future research directions, such as exploring their use for inhibiting the growth of pathogens affecting knee implants and extending the application of essential oils in coating titanium implant materials to enhance their resistance to failure.

In conclusion, it can be asserted that essential oils and their bioactive components can be quite helpful in the fight against germs that are resistant to antibiotics and may be effectively utilised in knee arthroplasty. This approach has the potential to reduce the need for revision surgeries resulting from illnesses resulting from bacteria resistant to antibiotics.The knowledge gathered from this research will add to the expanding corpus of knowledge in the health sciences regarding the efficacy of essential oils and their mechanisms of action against antibiotic-resistant pathogens.

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CHAPTER FIVE:
CONCLUSION AND FUTURE STUDIES

5.1. Introduction

Knee infections remain a crucial public health issue globally, particularly affecting disadvantaged local communities (Duque *et al*., 2023). The field of knee implantology has been developed to repair damaged knees; however, infections resulting from bacterial colonisation around the implant can lead to failure, either early or late after insertion. To mitigate these infections, antibiotic regimens are commonly employed; however, excessive antibiotic usage has resulted in the emergence of resistance bacterial strains (Setlhare, 2017; Kariminik *et al*., 2019). This growing resistance poses significant risks to patient health and contributes to escalating healthcare costs (Kariminik *et al*., 2019).

Natural products, like essential oils, have gained attention for their potential as therapeutic interventions because of their anti-diabetic, antioxidant, and antibacterial qualities. These compounds can effectively combat microbial species that exhibit resistance to conventional antibiotics (Magunga, 2016; Setlhare, 2017; Ntondini *et al*., 2021). Despite this potential, information about the impact of essential oils on human health remains limited (Ntondini *et al*., 2021). This lack of information necessitates further investigation into non-toxic yet effective microbial agents derived from fragrant plants traditionally used in remedies.

This study focused on evaluating the antibacterial properties of *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils against *S. aureus* and *S. epidermidis* associated with knee implants. By utilising a bioassay method, the research sought to detect the inhibiting impacts of these essential oils on bacterial growth and to assess the structural effects they exert on *S. aureus* and *S. epidermidis*. The findings indicate that both essential oils can be considered viable options among antibiotic-resistant microorganisms associated with knee implants.

5.2. Methodology and Key Findings

Gas chromatography-mass spectrometry (GC-MS) was employed to ascertain the chemical constituents accountable for preventing the development of microorganisms, as being aware of the chemical makeup of each essential oil is vital for elucidating their modes of action. Following GC-

MS examination, a bioassay was conducted in order to evaluate the ability of *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils to prevent the growth of antibiotic-resistant bacteria, specifically *S. aureus* and *S. epidermidis*. The results revealed significant antibacterial activity: *Melaleuca alternifolia* essential oil exhibited the largest inhibition zone of 39.1 mm against *S. aureus*, while *Pelargonium graveolens* essential oil showed an inhibition zone of 24.6 mm against *S. epidermidis*.

Subsequent microdilution assays were performed to determine the concentrations at which *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils stop the development of antibiotic-resistant *S. epidermidis* and *S. aureus*. The microdilution results showed that *Pelargonium graveolens* essential oil had a MIC value of 0.4 µg/mL against *S. aureus* and 0.2 µg/mL against *S. epidermidis*. Similarly, *Melaleuca alternifolia* essential oil exhibited an MIC value of 0.4 µg/mL against *S. aureus*. To further examine the structural alterations brought about by these essential oils, SEM and TEM were utilised, following the protocols established by Ncango *et al*. (2010).

The outcomes of this research provide compelling evidence of the antimicrobial effects of *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils, as observed through inhibition zones, MIC values, and structural alterations in the bacterial cells. This research supports the consideration of *Melaleuca alternifolia* and *Pelargonium graveolens* essential oils as viable agents that fight bacteria in the knee arthroplasty sector. The variations in the chemical makeup of the essential oils may account for their distinct effects on bacterial cells, warranting further investigation.

5.3. Future Studies

The current research opens several avenues for future studies, which may include:

- Investigating the biofilm formation of bacterial strains on the experimental knee implant
- Investigating the impacts of essential oils on human well-being and health

- Determining the optimal concentrations of essential oils that are safe for human cells while effectively eliminating bacteria
- Developing topical formulations, such as ointments containing essential oils, for individuals suffering from conditions like arthritis
- Exploring the adherence properties of essential oils on implant surfaces over time
- Identifying specific chemical components within essential oils that exert detrimental effects on bacterial cell walls and contents
    - Evaluating the essential oils' potential as cleaning agents for the surfaces of knee implants.

These future studies may contribute significantly to expanding knowledge about essential oils and how to use them in combating antibiotic-resistant diseases, thereby enhancing patient outcomes in orthopaedic care.

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