An investigation of the antimicrobial activity of essential oils against antibiotic resistant foodborne pathogens related to diarrhoea

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DECLARATION OF INDEPENDENT WORK

I, **Moleboheng Olga Mohlomi**, do hereby declare that this research project submitted to the **Central University of Technology, Free State** for the degree **MASTER OF HEALTH SCIENCES: ENVIRONMENTAL HEALTH** is my own work and has not been submitted before to any institution by myself or any other person in fulfilment of the requirements for the attainment of any qualification.

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Signature   Date

Central University of Technology, Free State

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SUMMARY

Infections that cause diarrhoea, which is a persistent health problem globally, are some of the most prevalent causes of mortality in children under the age of five. This health issue is also known to have caused as many as 500 000 deaths annually around the world (Najim et al., 2019). In children under the age of five, diarrhoea is known to spread through contaminated food, lack of access to safe drinking water, poor hygiene by mothers when handling paediatrics, non-breastfeeding or delayed breast-feeding, socio-economic status, and malnutrition (Akinyemi et al., 2018; Kuhn, 2019). Additionally, the Global Enteric Multicentre Study (GEMS) indicates that the rate of children presenting with diarrhoea which results in morbidity and mortality in developing countries is high (Liu et al., 2016 Aljamali, 2021). Most food poisoning reports (Aljamali, 2021) are associated with pathological defilement, especially among members of Gram-negative microbes like *Salmonella typhimurium, Escherichia coli* and *Pseudomonas aeruginosa* (Mostafa et al., 2018). Gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus cereus* have also been identified as causal agents of foodborne illnesses (Mostafa et al., 2018).

As severe diarrhoea has been categorised as life-threatening, scientists have endeavoured to discover antibiotic treatments to manage this threat. Initially, antibiotics were effective and reduced diarrhoeal cases among paediatric patients (Mantegazza et al., 2018; Najim et al., 2019). However, most microorganisms have developed resistance to antibiotics such as Cephalosporin, Methicillin, Tetracycline and Ampicillin, resulting in more problems in the medical field (Church & McKillip, 2021). Researchers have thus begun to search for alternative strategies to augment

the effect of currently used antibiotics, and one such alternative is the use of essential oils (Gibson et al., 2015; Chandra, 2017). The current investigation focused on the use of essential oils to address the effects of pathogens that cause diarrhoeal infection in children under the age of five. A point of departure was that antimicrobials with plant origins such as essential oils (EOs) are less likely to be associated with adverse side effects as they are natural products. Moreover, many such preparations have been used in foods such as spices and flavour enhancers and have been shown to have therapeutic potential to heal infectious diseases (Mehdi et al., 2018; et al., 2021).

To identify the constituents of the essential oils (also referred to as aromatic oils) that were used in the study, a gas chromatography-mass spectrometry (GC-MS) analysis was performed. The antimicrobial activities of the selected essential oils (*Helichrysum splendidum, Mentha piperita, Tagetes minuta, and Pelargonium graveolens*) that were used on the selected bacteria (*Escherichia coli, Clostridium difficile, Salmonella enterica, and Staphylococcus aureus*) were evaluated by using the bioassay and minimum inhibition concentration (MIC) methods. The analyses revealed that all four the selected oils contained components that are known to have antimicrobial effects on bacteria. However, the EOs that were tested affected bacterial cells differently per bacteria because of the difference in the bacterial cells of the microorganisms. For instance, *Tagetes minuta* and *Pelargonium graveolens* were the most effective oils as they showed greater zones of inhibition against the organisms. It is thus argued that these two oils show great promise for use as alternative antimicrobial agents

CHAPTER 1: ESSENTIAL OILS AS ALTERNATIVE ANTIMICROBIAL AGENTS AGAINST ANTIBIOTIC RESISTANT FOODBORNE PATHOGENS RELATED TO DIARRHOEA: BACKGROUND AND LITERATURE REVIEW

1.1. INTRODUCTION

Diarrhoea occurs when there is an infection in the gut or intestinal tract. It manifests as three or more loose stools a day that may last for up to fourteen days. It may be caused by a bacterium, a virus, or parasites (World Health Organization, 2017). Various studies have investigated the prevalence of diarrhoea in children under the age of five, and it seems highly likely that it spreads through contaminated food. It may also develop due to a lack of access to clean/healthy drinking water, poor hygiene practices by mothers, bottle instead of breastfeeding, and low socio-economic status (Akinyemi et al., 2018). Diarrhoea is rated as the second leading cause of death after pneumonia in children under the age of five, and it is considered a significant health issue globally (World Health Organization, 2005; Liu et al., 2016). It is also known to cause high annual mortality rates among young children worldwide (World Health Organization, 2005; Najim et al., 2019).

Escherichia coli, *Salmonella typhimurium*, *Campylobacter jejuni*, and *Shigella* are foodborne pathogens that have been implicated in several foodborne outbreaks that cause diarrhoea. The most common pathogen is *Escherichia coli* which is known to be a causative agent of bloody diarrhoea (referred to as haemorrhagic colitis) and renal failure (haemolytic uremic syndrome) in humans (Ban & Kang, 2016). Besides *Escherichia coli*, another foodborne pathogen that causes diarrhoea and physical signs of ill health such as belly pain, fever, chills, nausea, and vomiting is *Salmonella*, which is the causative agent of salmonellosis (Li et al., 2018).

Foodborne diseases are caused by the ingestion of food that is contaminated with microorganisms, which are mainly harmful bacteria. Other natural toxins such as moulds, viruses, and parasites, as well as chemical substances and physical agents, can also provoke infectious diseases (Ahmed et al., 2019). In some cases foodborne diseases are life-threatening, and this threat is especially prevalent among children living in developing countries and those with chronic diseases and disabilities. Children's symptoms can vary from light to severe and can manifest as abdominal colic, nausea, vomiting, dehydration, diarrhoea, and fever (Australian Department of Health, 2017; Cohen et al., 2019). Distinct contamination sources are polluted soil, poor food preparation, and inadequate food and meal storage practices (Kuhn, 2019). Despite many advances in the field of food safety and environmental health, billions of people worldwide are at risk of unsafe food (Fung et al., 2018).

1.2. FOODBORNE PATHOGENS CAUSING DIARRHOEA

According to a 2016/2017 World Health Organisation (2017) survey, unsafe food products that contained harmful bacteria, viruses, parasites, or chemical substances caused over two hundred diseases ranging from diarrhoea to cancers. Diarrhoeal diseases are the most common illnesses resulting from the consumption of contaminated food (World Health Organization, 2015a & b). A data synthesis that was conducted by Kirk et al. (2015) on the global and regional disease burden of 22 foodborne diseases estimated that 580 million such illnesses occurred in 2010. Norovirus alone was responsible for 125 million foodborne illnesses, which was the largest number caused by any pathogen. Other pathogens resulting in high numbers of foodborne cases were *Escherichia coli, Salmonella typhimurium*, *Staphylococcus aureus,* and *Clostridium difficile* (Franz et al., 2019).

1.2.1. *Clostridium difficile*

Clostridium difficile (*C. difficile*) is a Gram-positive spore-forming anaerobic bacterium that causes disease in humans and animals (Primavilla et al., 2019). This bacterium has for years been considered a nosocomial human pathogen that usually causes infectious diarrhoea in patients after hospitalisation and antibiotic treatment (Primavilla et al., 2019). *Clostridium difficile* is a common human enteric pathogen (Cimolai, 2019). The diseases it causes may vary from mild diarrheal symptoms to fulminant panniculitis, while many associated systemic manifestations ‐ including death ‐ have been detailed (Cimolai, 2019). In addition, *C. difficile* is also a major diarrhoea-causing pathogen in people of all ages and poses a significant threat to vulnerable and immunocompromised populations (Plants-Paris et al., 2019).

Clostridium difficile is a member of the *Clostridium* genus which is a large prokaryotic genus encompassing several clinically relevant species (Ramos & Rodloff, 2018). In 2007, *C. difficile*-causing pseudomembranous colitis contributed to the death of patients five times more often than MRSA (Ramos & Rodloff, 2018). Moreover, patients with *C. difficile* infection who were treated in intensive care units experienced a longer hospital stay and a higher mortality rate than uninfected controls (Ramos & Rodloff, 2018). According to Riley et al. (2019), an extended stay in a healthcare facility is associated with an increased risk of disease, and this is particularly linked with infection due to localised environmental contamination with metabolically inactive spores that are resistant to most cleaning protocols.

1.2.2. *Escherichia coli*

Escherichia coli (E. coli) is another vexing pathogen (Dewey-Mattia et al., 2017) that is associated with disease. For instance, in 2017 *E. coli* was responsible for 6% of the 902 foodborne disease outbreaks in the United States that were reported to the Centre for Disease Control and Prevention (CDC), rendering it the third largest source of foodborne illnesses in that year (Dewey-Mattia et al., 2017). This extracellular pathogen is transmitted from host to host via the faecal-oral route (Balière et al., 2015) through the ingestion of contaminated food and water as well as contact with contaminated surfaces (Yen et al., 2016; Vásquez-García et al., 2019). Hand washing is a fundamental precautionary measure to prevent the spread of disease and is one of the primary practices to reduce the transfer of bacteria. Therefore, if good hygiene is not practised, *E. coli* is highly likely to cause diseases (Lambrechts et al., 2014).

Enteric pathogenic *Escherichia coli* (EPEC) is considered one of the most predominant causative agents of diarrhoea in humans (Gaytán et al., 2016). It causes moderate to severe protracted diarrhoea that is accompanied by mild fever and sometimes vomiting (Vásquez-García et al., 2019). Among the bacteria that cause diseases, *Escherichia coli* has been identified as the most important agent for childhood diarrhoea in developing countries. For example, in Bangladesh 41-46% of acute diarrhoea was caused by *Escherichia coli* (Pervin et al., 2018).

EPEC causes severe diarrhoea in developing countries such as Brazil, Chile, Peru, and Mexico (Hu & Torres, 2015; Vásquez-García et al., 2019). Enteric pathogenic *Escherichia coli* colonizes the intestine epithelium, causing attaching and effacing (A/E) lesions that are characterised by effacement of the intestinal microvilli and the intimate attachment of the bacteria to the host epithelial surface, which is the central EPEC pathogenicity mechanism (Zhuang et al., 2017). These ultrastructural changes limit the absorptive capacity of the intestinal cells, leading to diarrhoea (Bhatt et al., 2016).

1.2.3. *Staphylococcus aureus*

Staphylococcus aureus is a zoonotic pathogen that can infect both humans and animals (Li et al., 2018). It is widely prevalent in nature where it is present in the air, water, and animal feed. It also exists on the surface of the human body, in the nasal cavity, on animal fur, and in the digestive tract, among other sites (Li et al., 2018). *Staphylococcus aureus* has been responsible for several infectious diseases such as tissue and skin infections, pneumonia, sepsis, mastitis, arthritis, and soft tissue infections (Li et al., 2018; Tong, 2015). Livestock products can act as a source of *S. aureus* zoonotic infections and handling or consuming such contaminated food could potentially result in the transmission to humans (Li et al., 2018). This bacterium also suppresses phagocytosis and cell-mediated immunity and produces an enzyme that inactivates most penicillin-based treatments. In recent years, the emergence and spread of antimicrobial resistant *S. aureus* strains, especially multidrug-resistant (MDR) strains, have become major public health concerns (Hoque et al., 2018).

1.2.4. *Salmonella typhimurium*

Invasive bacterial infections are a significant cause of morbidity and mortality among children in Southeast Asia (Emary et al., 2012; Najim et al., 2019). In this region, aggressive bacterial infections have been known to be a significant cause of morbidity and mortality among children under the age of five (Emary et al., 2012; Najim et al., 2019). Infections can be caused by several bacteria, with diarrhoea being among the most prevalent afflictions (Sattar et al., 2022). One of the causal agents of diarrhoea is *Salmonella typhimurium,* which is a major foodborne bacterium that is known to cause intestinal infectious diseases worldwide. This bacterium is resistant to multiple antimicrobial agents (Chen et al., 2021). For instance, isolates of *Salmonella typhimurium* resistant to multiple antimicrobial agents have caused epidemics and are endemic in many areas of south-eastern and southern Asia (Anwar, 2018). These include multidrug-resistant (MDR) isolates resistant to the previous first-line antimicrobials (chloramphenicol, ampicillin, co-trimoxazole) and those with intermediate susceptibility to ciprofloxacin (previously described as decreased ciprofloxacin susceptibility) (Emary et al., 2012; Najim et al., 2019).

Foodborne pathogens that cause diarrhoea are currently treated with antibiotics that are known to be successful in controlling infectious pathogens and increasing feed efficiencies (Mehdi et al., 2018). Antibiotics are thus routinely used to treat and prevent infections in humans and animals (Mehdi et al., 2018). However, scientific evidence suggests that the increased use of these compounds has intensified the problem of antibiotic resistance (Mehdi et al., 2018). For instance, the discovery and development of antibiotics such as penicillin during the 1900s gave a certain hope to medical science, but these antibiotics soon became ineffective against most bacteria (Chandra et al., 2017). A report by the Centre for Disease Control and Prevention estimated that more than two million individuals in the United States were susceptible to antibiotic resistant pathogens each year, resulting in nearly 23 000 fatalities (Harris et al., 2019).

1.3. ANTIBIOTICS

The discovery and development of antibiotics in the pharmaceutical industry in the twentieth century have been a most influential medical advancement (Uddin et al., 2021). Antibiotics have been of great benefaction to humans as they combat infections against bacteria and have saved millions of lives. However, with the advent of the twenty-first century, infections caused by multidrug-resistant (MDR) bacteria have been increasing around the globe (Uddin et al., 2021).

Today antibiotic resistance is a universal public health concern as it has implications for the successful treatment of human and animal infections (Mbanga et al., 2021). This problem has escalated in recent years and has resulted in clinical and economic impacts and health concerns globally. Most notably, it has caused healthcare-associated infections (nosocomial) resulting in death because of the increased difficulty in treating patients (Iseppi et al., 2021). Due to the escalation of resistance against most categories of antibiotics and increasing challenges to treat infections, the need for new strategies and antimicrobials has intensified (Iseppi et al., 2021). For this reason, the pharmaceutical industry and research communities have started showing interest in exploring the biodiversity of the world of plants and their derivatives as they contain important sources of molecules with a potentially broad spectrum of action. For example, essential oils extracted from aromatic plants have shown a wide range of antimicrobial activity against pathogens in various studies (Arena et al., 2018; Iseppi et al., 2021).

As antibiotic resistance has become a global problem, this calls for more research to identify alternative means to treat antibiotic resistant pathogens. Natural antimicrobials such as essential oils are reported to be effective in treating infections caused by antibiotic resistant pathogens, therefore this study focused on investigating the antimicrobial activity of essential oils on bacteria causing diarrhoea in children under the age of five.

1.4. ESSENTIAL OILS

1.4.1. Essential oils as potential antimicrobial agents

Essential oils are aromatic liquids that can be isolated from the natural secretions produced by various plant tissues. These liquids are stored within specialised secretory cells in these plants. The oils are commonly extracted by steam or a hydro-distillation process and their composition varies depending on climate, soil composition, plant organ, age, and vegetative life cycle (Boukhatem et al., 2013; Gucwa et al., 2018). Only 10% of plant species that are termed 'aromatic plants' produce these essential oils (Elshafie & Camele, 2017). The biological role of essential oils is to act as chemical signals through which the plant communicates with its environment to ensure proper development (Elshafie & Camele, 2017). These processes include the inhibition of the proximate growth of other plants and their subsequent germination while they also defend the mother plant against pathogenic microorganisms, herbivores, and insects (Elshafie & Camele, 2017). Due to their biological roles and aromatic nature, essential oils have numerous applications and are widely used in the food, sanitation, cosmetic, and perfume industries (Yancu & Sanderson, 2019). The term 'essential oil' refers to volatile oils that are stored in extracellular spaces in the epidermis or mesophyll cells of plants. Such oils have a low boiling point (Igwaran et al., 2017) and many plants that produce essential oils are used for medicinal purposes, mostly in traditional medicines. Several of these plants are also used in large volumes in the pharmaceutical, food, and cosmetics industries (Gucwa et al., 2018).

It has been reported that vegetables or greenery can generate antimicrobial constituents to protect themselves against biotic attacks, which is essential for resistance to microbial infections (Kutluk et al., 2018), while such plants are also known as affluent derivations of organically active compositions (Kutluk et al., 2018). Considering the global reality that numerous people in under-developed and developing countries have limited access to professional health services, plants with antimicrobial qualities are extensively used by traditional healers to treat infections and diseases (Kutluk et al., 2018).

Several researchers have demonstrated that EOs are effective against bacteria, yeasts, fungi, and parasites and that they are characterised by antioxidant, anti-inflammatory, and anti-carcinogenic properties (Giarratana et al., 2017; Arena et al., 2018; Chaaban et al., 2019). It has also been demonstrated that essential oils are more effective against Gram-positive bacteria than against Gram-negative bacteria, and their possible mechanism of action has been attributed to membrane permeability (Chandra et al., 2017).

According to Chandra et al. (2017), researchers are in search of novel antimicrobial agents that have a broad spectrum of activity against both Gram-negative and Gram-positive bacteria without having any side effects. Studies are therefore continuously conducted on a variety of medicinal plants in various countries. Moreover, the emergence of microorganisms that are resistant to nearly all classes of commercially available antimicrobial agents has become a driver for the discovery of new drug candidates (Kutluk et al., 2018). Many researchers argue that traditional healers have long used plants to prevent or cure infectious diseases, and scientific studies have subsequently revealed that a number of these antimicrobial agents have specific modes of action that are beneficial in this regard, such as disruption of the bacterial cell, leakage of the cell contents, and cell coagulation (Sahni et al., 2019).

Demands to minimise the use of antibiotics have intensified in recent years and have resulted in the search for alternative products to cure infections and diseases. The use of natural plant products, particularly those that produce EOs, has been considered because they are safe and limit risk to humans, animals, and the environment (Yulia, 2009). Furthermore, EOs are well known as antimicrobial agents that can be used to control food spoilage and foodborne pathogenic bacteria because they have a wide range of active chemical components such as Terpene hydrocarbons (monoterpenes and sesquiterpenes) and oxygenated compounds (phenols, alcohols, ketones, aldehydes, esters, lactones, oxides, ethers, and coumarins) (Burt, 2004; Chen et al., 2007). *In vitro* studies have shown that EOs have antimicrobial properties that are effective against Gram-positive bacteria, Gram-negative bacteria, moulds, and yeasts. Among the most studied bacteria are *Listeria monocytogenes, Salmonella typhimurium, Escherichia coli, Bacillus cereus*, and *Staphylococcus aureus* (Fisher et al., 2006; Amin et al., 2021). As essential oils have multifaceted antimicrobial effects, bacteria are less likely to develop resistance to them than to antibiotics (Cherrat et al., 2014). In this investigation, essential oils were thus used as potential antimicrobials to counteract the effect of pathogens that are associated with foodborne infections in humans.

To develop alternative antimicrobial drugs, the screening of various plants with the desired properties is now being conducted (Giarratana et al., 2017). The antimicrobial properties in plants are attributed to the presence of active compounds such as quinones, phenols, alkaloids, flavonoids, terpenoids, tannins, lignans, glucosinolates, and some secondary metabolites (Chandra et al., 2017). Other antimicrobial agents in plants include peptides that form a defence system that is similar to human antimicrobial peptides in structure and function (Chandra et al., 2017). Essential oils are also secondary metabolites in plants that have compounds with an isoprene structure, also known as terpenes, with the typical formula $C_{10}H_{16}$ (Chandra et al., 2017). Different types of terpenes are known such as diterpenes, triterpenes, and tetra terpenes, while hemiterpenes and sesquiterpenes have also been identified (Chandra et al., 2017). When compounds contain oxygen as an additional element, they are referred to as terpenoids, which are a different class of naturally occurring organic chemicals that are similar to terpenes (Chandra et al., 2017). Terpenes, or terpenoids, are active against bacteria, fungi, viruses, and protozoa (Chandra et al., 2017). These are potential alternatives to synthetic insecticides because of their limited persistence in the environment and a low probability that they will generate resistance (Arena et al., 2018).

Many plant species are of commercial interest because they can produce essential oils. The use of essential oils for both spiritual and health reasons has a long history (Ghavam et al., 2021) and it is well known that plant products are the principal sources of pharmaceutical agents in traditional medicines (Ghavam et al., 2021). Some medicinal plants are rich in antimicrobial agents, and it is widely accepted that this ability is derived from essential oils that possess insecticidal, antifungal, anti-inflammatory, and antioxidant properties (Lin et al., 2016).

1.4.1.1. Tagetes minuta essential oil

Extracts from plants of the *Tagetes* species, which belongs to the family Asteraceae, are used as traditional remedies in many countries to treat colic, diarrhoea, vomiting, fever, skin diseases, and hepatic disorders (Giarratana et al., 2017). Moreover, *Tagetes minuta* essential oil has substantial antibacterial activity against both Gram-negative and Gram-positive bacteria (Santos et al., 2017)

Other than *Tagetes minuta*, *Tagetes patula* L. (Asteraceae) is popularly known as dwarf- or French marigold. It is an annual plant, grows 20-30 cm in height, is native to North America, and has been widely disseminated throughout the world. The genus *Tagetes* is known to have biological effects against organisms such as fungi, Gram-positive and Gram-negative bacteria, viruses, nematodes, insects, ticks, and others (Politi et al., 2016). Phytochemical investigations of different parts of *Tagetes patula* have resulted in the isolation of several chemical constituents of different classes of secondary metabolites such as benzofurans, carotenoids, flavonoids, and thiophenes. The latter is responsible for a variety of biocide properties (Politi et al., 2016). Moreover, because of its constituents, this essential oil can act as an antimicrobial agent against *Staphylococcus aureus, Enterobacter cloacae, Escherichia coli,* and *Mycobacterium smegmatis* (Aboi et al., 2017). A native from the Americas, *Tagetes minuta L*. is already widely used in the agriculture, food, and pharmaceutical industries (Lovatto et al, 2013; Chaaban et al., 2019). Popularly known as Wild Marigold, *T. minuta* is a spontaneous-growth aromatic weed plant that reproduces by seeds (Wanjala & Wanzala, 2015; Chaaban et al., 2019).

1.4.1.2. ***Helichrysum* essential oil**

The name *Helichrysum* is derived from the Greek words ‘helios’ that means sun and ‘chrysos’ that means gold. The name refers to this flower’s colours as defined in Theseus (Kutluk et al., 2018). Members of this genus may be annuals, herbaceous perennials, or shrubs, and some species can grow up to 90 cm tall. There are over 600 species of *Helichrysum* worldwide but they occur mostly in Africa, Madagascar, Australasia, and Eurasia (Kutluk et al., 2018). As members of the Asteraceae (Composite) family, *Helichrysum* species are widely acknowledged as traditional medicinal plants. There are 20 species in Turkey, but more than 244 species have been identified in South Africa (Kutluk et al., 2018) and decoctions of various *Helichrysum* species have been widely used in Turkish and South African folk medicine. Traditional preparations mainly consist of decoctions for their stomachic, diuretic, and anti-asthmatic properties while they are also in demand for their ability to break down kidney stones (Kutluk et al., 2018).

In 2007, Appendino and colleagues reported that *Helichrysum italicum* was well documented in terms of ethnobotanical use and clinical efficiency and they performed a systematic evaluation of the plant as a source of new pharmaceuticals (Kutluk et al., 2018). It has also been demonstrated that alpha-pinnene and beta-pinnene found in *Helichrysum* oil can destroy cellular integrity and that they are effective against *S. aureus and E. coli* (Mastelic et al., 2005; Kutluk et al., 2018). Microscopic investigations showed a frequent occurrence of disorganisation of reproductive organs, the division of the cell membrane of the cell wall, and destruction of cellular energy-generating structures of bacteria when using *Helichrysum* oil (Mastelic et al., 2005; Kutluk et al., 2018).

1.4.1.3. *Mentha piperita* essential oil

High or increased phagocytic activity against various pathogens has been reported in fish after treatment with different types of medicinal plants, including *Mentha piperita* (peppermint). This is one of the most widely used herbs worldwide as it has a long history of safe use in medicinal preparations (Ribeiro et al., 2018). The use of *Mentha piperita* is promising as it may offer a low-cost alternative for use in the medicine and food industries (Adel et al., 2015; Ribeiro et al., 2018).

Commercially, EOs are important due to the presence of high-value monoterpenes such as menthol and methane (Wanke et al., 2015). Generally, the most important mint species are peppermint (*Mentha piperita* L.), menthol mint (*Mentha arvensis* L.), and spearmint (*Mentha spicata* L.) (Machiani et al., 2018).

Countries that cultivate this aromatic plant are Bulgaria, Italy, China, and the USA, which are responsible for about 90% of the world's total peppermint essential oil production (Machiani et al., 2018). The global demand for peppermint essential oil has increased in recent years (Machiani et al., 2018), particularly as peppermint essential oil has anticancer, antidiabetic, antimutagenic, antibacterial, antifungal, and antiviral properties (Machiani et al., 2018). Menthol and menthone are the most important volatile constituents in peppermint and other Mentha species and are known for their typical cooling minty taste and smell (Tiwari, 2016; Zheljazkov et al., 2016; Wanke et al., 2015). Usually, peppermint essential oil contains 30-55% menthol and 14-32% menthone (Machiani et al., 2018). The quality of peppermint essential oils depends on the percentage of menthol, menthone, menthofuran, and pulegone they contain and they are improved by increasing the menthol and menthone percentages and decreasing menthofuran and pulegone (Wanke et al., 2015). Some studies demonstrated that agricultural and environmental factors such as long days and cool nights and nutrient availability during the growth period could improve the quality and quantity of essential oil-bearing plants (Verma et al., 2016; Machiani et al., 2018).

1.4.1.4. *Geranium* essential oil

Geranium *(Pelargonium graveolens)* is a redolent herb that yields geranium oil. The effect of geranium oil on human wellbeing and mental health has been published by numerous scientific studies (Hassanvand et al., 2019). Due to its extraordinary characteristics, the wide utilization of geranium oil as an herbaceous element in fragrances and cosmetics has been prevalent in recent years. Geranium oil is also a principal ingredient in the soap industry and is extensively utilized in the pharmaceutical industry, in herbal medicines, and in aromatherapy. Hassanvand et al. (2019) argue that both the rise of essential oil prices and the increase in market demand for such products call for the maximization of essential oil production (Hassanvand et al., 2019).

Pelargonium graveolens, popularly known as rose-scented geranium, belong to the Geraniaceae family. It is a fragrant bushy plant that grows up to 1 m in temperate, subtropical, and tropical climates and in a wide variety of soils (Silva et al., 2017). A light yellow, volatile oil is obtained from the leaves, with geraniol and citronellal as the main constituents. The essential oil of rose-scented geranium is also widely used as a substitute for rose scents in the perfumery and cosmetics industries (Silva et al., 2017). Moreover, the oil possesses antioxidant and antimicrobial properties and is an important source of active ingredients in the pharmaceutical industry (Bigos et al., 2012; Silva et al., 2017).

Rose geranium (*Pelargonium radens* x *Pelargonium capitatum*) is an essential oil plant that belongs to the Geraniaceae family. The *Pelargonium* species originates from the southern tip of Africa and was introduced to Britain and Holland through the spice trade while sailors also collected it as a medicinal plant treatment (Miller, 2002). Weiss (1997) indicates that the first *Pelargonium* (*P. cucullatum*) was collected from Table Mountain in 1672, which makes South Africa the centre of the origin of this genus. Of 25 *Pelargonium* species, only four are important for the production of essential oils, namely *P. graveolense*, *P. odoratissium*, *P. capitatum,* and *P. radens*.

1.4.2. Mechanism of action of essential oils against bacteria

In elucidating the mechanism of action of EOs on bacterial cells, various mechanisms have been described (Abers et al., 2021). This mechanism is related to the structure of the cell wall, the membrane permeability of bacteria, different bacterial architectures (such as Gram-positive and Gram-negative bacteria that differ in their cell membrane compositions, the chemical composition of the EOs, and the quantity of the major single compounds (Nazzaro et al., 2013; Swamy et al.,2016; Gucwa et al., 2018). Each compound may exhibit a different mechanism of action against the microbes they encounter (Swamy et al., 2016). Moreover, the antimicrobial action of EOs is linked to one of their most important characteristics, which is their hydrophobicity that results in increased cell permeability and consequent leaking of cell constituents (Faleiro, 2012). Overall, the mechanism of antibacterial action is mediated by a series of biochemical reactions in the bacterial cell, and these reactions are dependent on the type of chemical constituents present in the essential oil (Swamy et al., 2016).

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It is noteworthy that a disturbed cell structure may affect other cellular structures in a cascade type of action (Faleiro, 2012). The activity of an EO can therefore affect both the external envelope of the microbial cell and the cytoplasm (Nazzaro et al., 2013). The hydrophobicity that is typical of EOs is thus responsible for the disruption of bacterial structures and this leads to increased permeability due to an inability to separate the EOs from the bacterial cell membrane (Nazzaro et al., 2013). Thus, the hydrophobic nature of EOs allows them to penetrate microbial cells and cause alterations in their structure and functionality (Nazzaro et al., 2013).

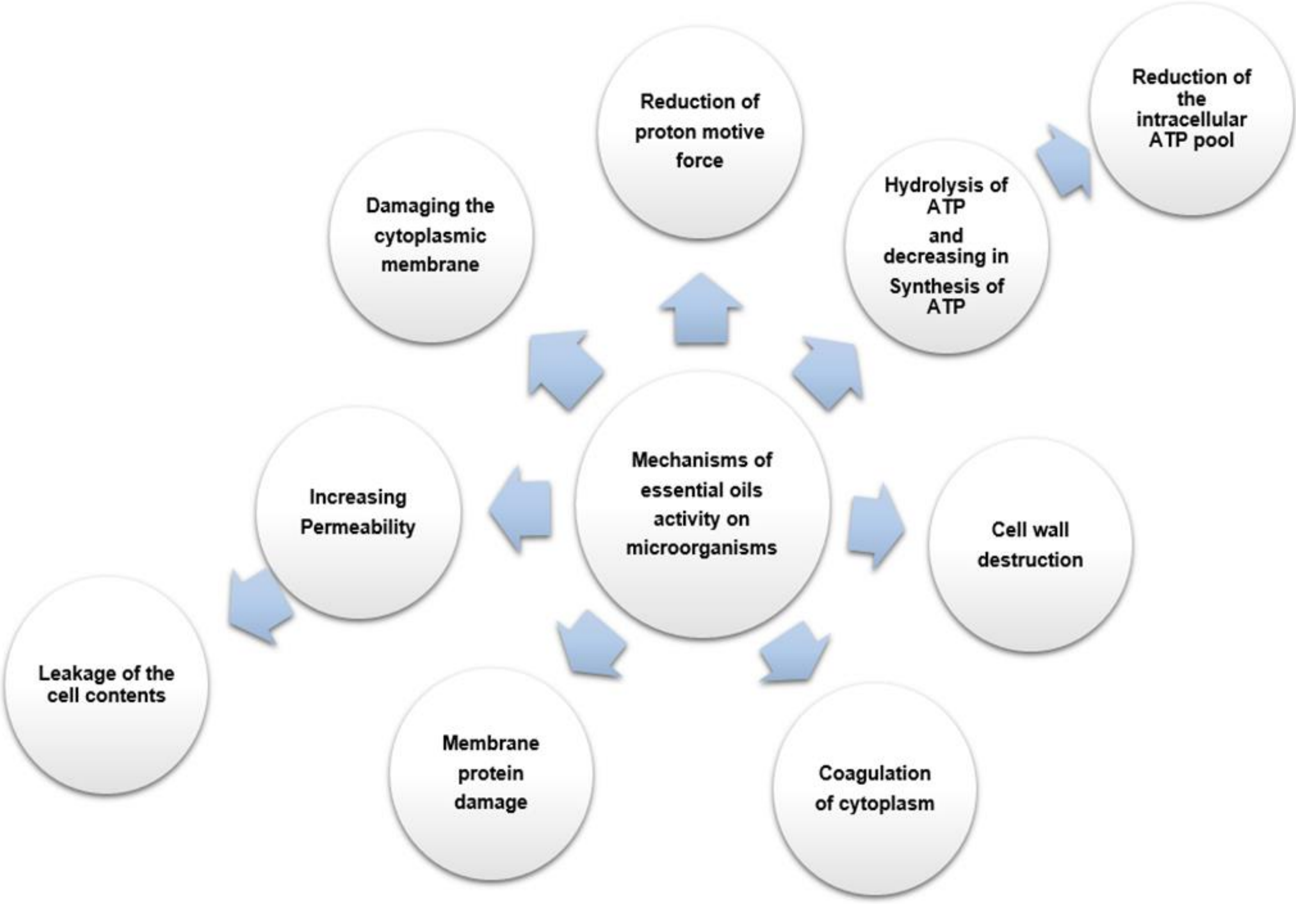

Figure 1: Mechanism of activity of Essential oils on microorganisms

Source: (Bhavaniramya, et al., 2019).

The impacts of EO mechanisms are listed in **Figure 1**. These are degradation of the cell wall, damage to the cytoplasmic membrane, cytoplasm coagulation, damage to membrane proteins, increased permeability leading to leakage of the cell contents, reduction of the proton motive force, reduction of the intracellular ATP pool via decreased ATP synthesis that is augmented by hydrolysis that occurs separate from increased membrane permeability, and reduction in the membrane potential via increased membrane permeability (Nazzaro et al., 2013). This latter phenomenon indicate that the bacterial membrane is the first target of essential oils (Nazzaro et al., 2013; Hassanvand et al., 2019).

1.5. RATIONALE

1.5.1. Problem statement

According to the Global Enteric Multicentre Study (GEMS), the rates of children presenting with diarrhoea that result in morbidity and mortality in developing regions such as sub-Saharan Africa and South Asia are extremely high (Liu et al., 2016). Children are mostly infected by consuming contaminated food, and this is due to incorrect food handling practices (Kotloff et al., 2013). Each year about 1.7 billion cases of diarrhoea are reported and, of these, about 578 000 deaths occur. Moreover, diarrhoea has caused an increased mortality rate in children under the age of five (Liu et al., 2016). Factors that contribute to the occurrence of diarrhoea in children vary due to the interaction between socio economic, environmental, and behavioural variables (Ahmed et al., 2019). Risk factors leading to infections that cause diarrhoea include a young age, malnutrition, early weaning, seasonal patterns, low maternal education, lack of potable water, lack of vigilant hand washing, and poor sanitation (Australian Department of Health, 2017). These challenges are compounded by the emergence of antibiotic resistant pathogens, and the widespread occurrence of resistant bacteria that lead to many health-related problems is now very common (Chandra et al., 2017). According to the World Health Organization (2017), resistance is highly prevalent in cases of bacterial infections that target the respiratory tract and that manifest as diarrhoea, meningitis, syphilis, gonorrhoea, and tuberculosis. For instance, *Staphylococcus aureus* isolated from clinical samples has shown resistance to more than three drugs and is considered a multiple-drug resistant bacterium (Chandra et al., 2017; Ejaz et al., 2020).

Bacterial resistance to drugs is a disconcerting public health issue considering its associated deleterious consequences. Moreover, misuse of antibiotics promotes antibiotic resistance, and this is responsible for an estimated 700 000 deaths annually worldwide (Silva et al., 2019). In France alone, 12 500 such deaths occurred in 2014/2015 (Colomb-Cotinat et al., 2015). Antibiotic misuse can also lead to increased duration of hospitalisation, lengthy healthcare requirements, and long periods of time off work due to persistent infections (Silva et al., 2019). In 2017, the World Bank published an evaluation of the economic impact of antibiotic resistance. The report stated that, "as antibiotic resistance spreads, healthcare expenditures will increase in step with the rising disease burden; by 2050 the annual costs may exceed the base-case level by 6% in high-income countries" (Silva et al., 2019). In light of the socioeconomic impact of antibiotic misuse and its consequences, devising methods to assess the costs associated with such misuse is important (Silva et al., 2019).

Antibiotics are the most widely prescribed drugs in the paediatric population and are administered to more than 50% of patients between birth and 18 years of age (Gibson et al., 2015; Mantegazza et al., 2018). Researchers have strongly urged that antibiotic therapy must be used with caution as it may result in a wide range of adverse events, including antibiotic-associated diarrhoea (AAD) (Mantegazza et al., 2018). The excessive use of antibiotics has been confirmed as a factor that contributes to an increase in drug resistance (Chandra et al., 2017). Moreover, most oral and intravenous antibiotics are associated with diarrhoea in children (Gibson et al., 2015; Mantegazza et al., 2018), and the risk of AAD is higher when antibiotics are administered against anaerobes (Mantegazza et al., 2018). Another issue is that many people use antibiotics without knowing the importance of completing the full course. After taking two to three doses of a particular antibiotic, many people feel better and then discontinue the course (Chandra et al., 2017).

Chandra et al. (2017) argue that there is an urgent need for medical assistance in treating these resistant bacteria and their effects. In comparison to first-line drugs, second- or third-generation drugs are much costlier and, due to a lack of financial assistance, many diseases cannot be treated efficiently in this way any more (Chandra et al., 2017). The ascending incidences of multidrug resistance to pathogenetic microorganisms have compelled the need to research different antimicrobial sources (Santos et al., 2017). The severity of drug resistance is an alarming phenomenon for scientific and medical professionals who argue that there is a need to search for alternative treatments or the development of new drugs to combat drug-resistant bacteria (Chandra et al., 2017). In light of the foregoing, the current study investigated the antimicrobial action of essential oils on pathogens that cause infections in humans. In this investigation it was acknowledged that natural or plant-sourced antimicrobials are not related to many side effects and that they have the therapeutic potential to heal infectious diseases (Mehdi, et al. 2018). It was also confirmed in the literature review that antibiotic resistant pathogens have emerged as a major threat to public health (Klevens et al., 2007; Mehdi et al., 2018).

The current research investigated the antimicrobial activity of selected essential oils, namely *Tagetes minuta, Helichrysum italicum, Mentha piperita, and Pelargonium graveolens* against selected foodborne pathogens (*Clostridium difficile*, *Escherichia coli, Salmonella typhimurium, Staphylococcus aureus*). It was argued that these pathogens cause diarrhoea in children under the age of five (Ashraf, 2016).

1.5.2. Aim of the study

The overarching aim of the study was to investigate the activity of essential oils to determine their effect on selected pathogens that are notoriously foodborne and that cause diarrhoea.

1.5.3. Objectives of the study

The objectives of the study were the following:

- Characterise the essential oils *Tagetes minuta, Mentha piperita, Helichrysum splendidum, and Pelargonium graveolens* using gas chromatography-mass spectrometry (GC-MS);
- Determine the minimum inhibitory concentrations (MIC) of the selected essential oils that will impact the organisms *Clostridium difficile*, *Escherichia coli*, *Salmonella*, and *Staphylococcus aureus,* and to evaluate their inhibitory effect on these organisms using the bioassay method; and
- In assessing the mode of action of the selected essential oils, determine the effect of the essential oils on the cell morphology of the selected bacteria using scanning electron microscopy (SEM).

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CHAPTER 2: ANTIMICROBIAL ACTIVITY OF ESSENTIAL OILS AGAINST SELECTED FOODBORNE PATHOGENS: MATERIALS AND METHODS

2.1. Abstract

Aims: To investigate the antimicrobial activity of four commercially accessible plant-derived essential oils, namely *Helichrysum splendidum, Mentha piperita, Tagetes minuta,* and *Pelargonium graveolens,* to determine their effect on antibiotic resistant bacteria that are known to cause infections that result in diarrhoea. These bacteria were: *Escherichia coli, Clostridium difficile, Salmonella enterica,* and *Staphylococcus aureus*.

Materials and methods: Essential oils consist of different components that are responsible for inhibiting bacterial growth. Gas chromatography–mass spectrometry (GC-MS) was conducted to analyse the chemical composition of the four selected essential oils. Next, the bioassay method was used to assess the antimicrobial action of these essential oils. The broth microdilution method was used to assess the minimum inhibitory concentrations (MICs) of the different essential oils to determine their effect on the selected bacteria. Afterwards, scanning electron microscopy (SEM) was utilised to evaluate the structural changes of bacterial cells after the application of the essential oils.

Results: The analysis of the selected essential oils revealed that all four contained components that are known to have an antimicrobial effect on bacteria. However, *Pelargonium graveolens* and *Tagetes minuta* were the most effective essential oils as they showed promising results when evaluated using bioassay, MIC, and SEM. These essential oils greatly affected the *Escherichia coli* cell wall as they caused it to develop holes, incomplete cell division, and eventually to die.

Keywords: Essential oils; foodborne pathogens; antimicrobial activity; inhibitory effect.

2.2. INTRODUCTION

Resulting from bacterial infections of the gut and/or the intestinal tract caused by food or water contamination, diarrhoea can ensue in three or more free stools that can last for up to fourteen days. Such infections can be caused by organisms such as bacteria, a virus, and parasites (World Health Organization, 2017). In humans, and especially in children below five years of age or younger, diarrhoea develops due to contaminated meals, a lack of potable water, poor hygiene practices, non-breastfeeding (or delayed breastfeeding), and malnutrition (Akinyemi et al., 2018). Rated as the second main cause of death in the paediatrics field, the causes of diarrhoea are a critical fitness/health problem globally (World Health Organization, 2005; Liu et al., 2016). Such infections cause an estimated 500 000 deaths worldwide every year (World Health Organization, 2005), while Liu et al. (2017) and Najim et al. (2019) state that they caused about 296 000 deaths in children younger than five years in 2015 in sub-Saharan Africa.

Escherichia coli, *Salmonella typhimurium*, *Campylobacter jejuni*, and *Shigella* are principal foodborne pathogens that have been identified in several foodborne outbreaks (Balière et al., 2015; Demissie et al., 2021). The most widespread of these is *Escherichia coli* which is known to inflict severe infection that causes bloody diarrhoea (haemorrhagic colitis) and renal failure (haemolytic uremic syndrome) in human beings (Ban & Kang, 2016). Apart from *Escherichia coli*, other pathogens that cause diarrhoea are *Salmonella*, the causative agent for salmonellosis, *Staphylococcus aureus*, and *Clostridium difficile* (Li et al., 2018).

As infections that cause diarrhoea are life-threatening in children under the age of five, the need to develop a cure to treat and control infections that cause diarrhoea is widely recognised (Khalil et al., 2018). Although the use of antibiotics to treat diarrhoea or any infection caused by bacteria has been successful for many years (Mantegazza et al., 2018; Najim et al., 2019), pathogenic organisms have managed to develop resistance against antibiotics (Mantegazza et al., 2018) and researchers have started to search for alternative treatments to curb the impact of bacteria (Gibson et al., 2015; Chandra et al., 2017). The severity of drug resistance is an alarming scenario for scientific and clinical professionals and they thus recognise the need to search for alternative remedies or enhance new antimicrobials to fight drug resistant microorganism (Chandra et al., 2017). This investigation focused on the antimicrobial effect of essential oils on pathogens that cause infections that result in diarrhoea. Antimicrobials with plant origins such as essential oils are currently not related to negative outcomes in humans, and it has been widely published that they have a therapeutic ability to heal infectious illnesses (Mehdi et al., 2018).

2.2 MATERIALS AND METHODS

2.2.1. Microorganisms

The bacterial strains used for this study were obtained from Thermofischer Scientific: Microbiology division in Randburg. These strains were *Escherichia coli, Clostridium difficile, Salmonella enterica* and *Staphylococcus aureus*. The microorganisms were cultured on plate count agar (PCA) at 37°C (Setlhare, 2017; Mostafa et al., 2018). The cultures were grown for 24 hours at 37°C on PCA.

2.2.2. Antibacterial products and their composition

2.2.2.1. Essential oil characterisation

Gas chromatography in conjunction with mass spectrometry (GC-MS) detection was carried out to analyse the composition of the selected essential oil samples. The essential oils that were used were *Helichrysum splendidum, Mentha piperita, Tagetes minuta,* and *Pelargonium graveolens*. They were characterised using gas chromatography that was equipped with an AB-1MS (30 Mx0.25 mm id 0.25 μm) capillary column (Kirbaslar et al., 2009; Adams, 2012; Igwaran et al., 2017). To prepare samples for analysis, the EOs were dissolved in hexane (10%) followed by an injection at a split ratio of 50:1 in a Finnegan Focus Gas Chromatograph that was set at 230˚C. At an injection temperature of 25°C, Helium was used as a carrier gas at a constant flow of 1 $mLmin^{-1}$ (at a split ratio of 50:1). The chromatograph oven was optimized at an initial temperature of 40˚C for 4 minutes and then raised to 5˚$Cmin^{-1}$ to 200˚C. This was held at 200˚C for 1 minute and then raised by 5˚C to 220˚C where it was held for 10 min. Mass analysis of the oils was done using a Finnegan Focus DSQ mass spectrometer. The ion source temperature was set at 250˚C with an ionization voltage of 70 eV and mass scan range of 50-650 amu. The identification of the compounds followed the procedure as proposed by Kirbaslar et al. (2009). Individual GC peaks and mass spectra were determined by searching commercial libraries followed by expert matching of MS data (Kirbaşlar et al., 2009).

2.2.3. Bioassay preparation

The antimicrobial activity of the selected essential oils and ethanol (96%) was used as a negative control and was determined by bioassay (Ghabraie, 2014) based on the agar diffusion method for infection-causing bacteria (*Clostridium difficile, Escherichia coli, Salmonella enterica,* and *Staphylococcus aureus*). After 24 hours of cultivating organisms, a final bacterial inoculum of $5x10^5$ CFU.ml^{-1} was prepared using Mueller-Hinton Broth (MHB). Then 200 µl of the organism was taken from the bottles and transferred into the prepared petri dishes containing a plate count ager (0.5% m.v-1 agar) and spread over the plate using a 'hockey stick' glass rod. A well (0.5 cm in diameter and depth) was then constructed at the centre of the plate containing an organism, and 46 µl of essential oil was added into the well. Ethanol (96%) was used as control and was added separately to the wells of the prepared plates. All the plates were incubated at 37°C for 24 h. To prevent evaporation of the essential oil from the plate, the oil was permitted to disperse in the agar before incubation.

2.2.4. Inhibitory effect of essential oils on bacteria

To determine the inhibitory effect of the selected essential oils on the selected bacteria, a broth microdilution method (Shawish et al., 2014) was executed using a 96-well disinfected microtiter plate. A final bacterial inoculum of $5x10^5$ CFU.ml^{-1} was developed using Mueller-Hinton Broth (MHB) and reciprocated into a 96-well sterile microtiter plate (Becton, Dickinson and Company, Sparks, USA). The analysed essential oil samples were added to the first row of wells and serial dilutions were performed to achieve final concentrations of 100, 50, 25, 12.5, 6.3, 3.1, 1.6, 0.8 $mg.ml^{-1}$. After infusion, the plates were incubated at 37°C for 24 hours. This analysis process was run twice in parallel for each concentration. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of an antimicrobial agent that prevents the visible growth of a microorganism in a broth dilution susceptibility test. The minimum inhibitory concentration was characterised in the first well without visible growth of bacteria.

2.2.5. Scanning Electron Microscopy (SEM)

To assess if structural changes occurred in the bacteria due to the function of the essential oils, scanning electron microscopy (SEM) was performed as proposed by van Wyk and Wingfield (1991). Briefly, throughout the bio-assay preparatory process, cells susceptible to two oils (*Tagetes minuta* and *Pelargonium graveolens*) and a control specimen were fixed using 3% v/v of a sodium phosphate buffered glutaraldehyde solution at pH 7.0 and a similarly buffered solution (1% m.v-1) of osmium tetroxide for 1 h. Subsequently, the material was dehydrated in a graded series of the ethanol solution (30%, 50%, 70%, 90%, and 100%) for 30 min per solution). Thereafter, ethanol-dehydrated material was critical-point dried, mounted, and coated with gold to make it electrically conductive. This preparation for each sample was examined using a scanning electron microscope (SEM).

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CHAPTER 3: RESULTS AND DISCUSSION

3.1. Introduction

Essential oils are extracted from various aromatic plants that are commonly found in warm countries (Lin et al., 2016). These plants are known for their antioxidant, antiseptic, medicinal, and fragrance properties and are also often used in the preservation of foods and as analgesics, sedatives, anti-inflammatories, and spasmolytic and local anaesthetics (Nazzaro et al., 2013; Hassanvand et al., 2019). Essential oils also contain a wide series of secondary metabolites that can inhibit or slow the growth of bacteria, yeasts, and moulds (Nazzaro et al., 2013; Chandra et al., 2017).

The biological activities of EOs correlate with the presence of specific chemical components as well as the ecological conditions of the countries where they occur. They vary from region to region and could have a direct influence on the chemical composition of other plant materials due to the accumulation of some of their components at a particular period in response to environmental conditions. This phenomenon in such plants could exhibit different biological and antioxidant activities (Moghaddam & Mehdizadeh, 2017). Furthermore, the hydrophobicity and components of essential oils enable them to penetrate the lipid bilayer of bacterial cell membranes and mitochondria, and this disrupts the targeted cell structure and renders them more permeable which, in turn, results in the leakage of critical molecules from within the bacterial cell and, eventually, its death (Omayma et al., 2020). The ability of some essential oils to insert themselves into the phospholipid bilayer of bacterial cell walls, where they bind to proteins and prevent them from performing their normal functions, is due to their richness in phenolics (Nazzaro et al., 2013; Yancu & Sanderson, 2019). This phenomenon indicates that the cell membrane is the first target of EOs. As previously reported, the mechanism of action of an EO is not an isolated activity but involves a series of events that occur both on the targeted cell surface and within the cytoplasm (Omayma et al., 2020).

The versatility of EOs is attributed to a strand of properties such as their chemical composition and their antimicrobial capacity. These are not attributable to a unique mechanism but are a cascade of reactions involving the entire bacterial cell. This may be because of the wide variety of molecules present in these natural extracts (Omayma et al., 2020). Additionally, as essential oils and their constituents have a range of targets/quarries ‐ particularly the membrane and cytoplasm of a cell ‐ they will, in certain conditions, comprehensively convert the morphology of the cells they target (Nazzaro et al., 2013; Hassanvand et al., 2019).

The structure of a Gram-positive bacterium cell wall allows hydrophobic molecules to easily penetrate the cell and act on both the cell wall and within the cytoplasm. Phenolic compounds, which are also present in EOs, generally show antimicrobial activity against Gram-positive bacteria. Their effect depends on the quantity of the compound present: at low concentrations they can interfere with enzymes involved in the production of energy, and at higher concentrations they can denature proteins (Nazzaro et al., 2013; Omayma et al., 2020). Furthermore, the sustained loss of ions or metabolites due to exposure to an essential oil can compromise the microbial metabolism and lead to cell death. For example, tea tree oil can cause *E. coli* to die without lysing the cell (Mostafa et al., 2018; Yancu & Sanderson, 2019). In this study, the antimicrobial activity of essential oils was investigated and evaluated through the agar diffusion and micro dilution methods and by analysing the morphological changes caused by the essential oils on the organisms using electron microscopy.

3.2. The Chemical Composition of the Selected Essential Oils

The essential oils used in the study were first characterised to evaluate their composition. This was done using gas chromatography mass spectrometry method. **Table 1** below presents the compounds that were identified in the essential oils that were used in this study. Most of the compounds were terpenes which are known to possess antimicrobial properties (Omayma et al., 2020).

Helichrysum splendidum was clear to pale yellow. It was aromatic with a characteristic herbal odour with a slightly sweet note. It was made up of compounds representing 95.84% of the total oil with p-Cymene (5.44%), Eucalyptol (1,8-Cineole) (15.9%), α-Pinene (6.28%), α-Phellandrene (5.63%), and β-Pinene (36.76%) being the main components of the oil. This was contrary the compositions in a study by Mashigo et al. (2015), who argue that the concentrations of constituents in EOs tend to vary due to environmental differences as well as seasonal changes.

Similar to the findings of Ejaz et al. (2020), *Mentha piperita* was characterised by a clear, pale-yellow liquid with a mint odour and 11 components that made up 89.79% of the oil, with 1,8-cineole (eucalyptol) menthofuran (12.57%), menthol (33.05%), menthone (24.02%), and menthyl acetate (8.90%) being the main compounds. *Tagetes minuta* was a clear, pale-yellow colour with an aromatic herbal odour and a slightly sweet note. It had 15 compounds representing 93.11% of the oil with four main constituents: p-Cymene 1,8-cineole (eucalyptol) (3.30%), 2-Bornanone (camphor) (1.15%), trans-Thujone (β-thujone) (14.45%), and α-Thujene (71.59%). This oil demonstrated antimicrobial activity in a previous study (de Olanda et al., 2019).

The following were revealed as the main compounds in *Pelargonium graveolens:* Citronellyl acetate (19.45%), Citronellol (24.28%), Geraniol (17.83%), and 2-Phenylethanol (12.54%). These were similarly indicated by Okla et al. (2022), who found that *Pelargonium graveolens* had geraniol and citronellol which are known to be active compounds in deactivating microbes such as *Escherichia coli* and *Salmonella typhimurium* as they cause damage to cellular membrane integrity and function.

Table 1: Combination of chemical properties of EOs (*Helichrysum splendidum, Mentha piperita, Tagetes minuta, Pelargonium graveolens*) used in the current study.

	Essential Oils (%)			
Compounds	*Helichrysum splendidum*	*Mentha piperita*	*Tagetes minuta*	*Pelargonium graveolens*
1,8-cineole (eucalyptol)	15.79	5.01	3.30	-
2-Bornanone (camphor)	0.18	-	1.15	-
menthofuran	-	12.57	-	-
menthol	-	33.05	-	-
menthone	-	24.02	-	-
menthyl acetate	-	8.90	-	-
trans-Thujone (β-thujone)	-	-	14.45	-
α-Phellandrene	5.63	-	-	-
α-Pinene	6.28	-	0.06	-
α-Thujene	0.31	-	71.59	-
β-Pinene	36.76	-	0.11	-
Citronellyl acetate	-	-	-	19.45

Citronellol	-	-	-	24.28
Geraniol	-	-	-	17.83
2-Phenylethanol	-	-	-	12.54

3.3. Antimicrobial Activity of Essential Oils

After analysing the chemical composition of the selected essential oils, an antimicrobial activity test was conducted using the agar dilution method referred to as a 'bioassay' method (Magunga, 2016; Mostafa et al., 2018). The different inhibition zones observed on each organism treated with different essential oils, revealed the ability of essential oils to inhibit the growth of these organisms. According to the results obtained, an undiluted *Pelargonium graveolens* showed greater inhibition zones against *Escherichia coli* when compared to other organisms (*Staphylococcus aureus, Salmonella typhimurium, and Clostridium difficile*) with a diameter of 37mm as shown in **Figure 2**. However, diluted *Pelargonium graveolens* showed only a 5 mm inhibition zone against *Escherichia coli*, whereas diluted *Pelargonium graveolens* showed a greater inhibition zone compared to that of *Staphylococcus aureus* at 19 mm diameter.

Moreover, with undiluted *Tagetes minuta*, the bioassay revealed an inhibition zone of 29 mm against *Clostridium difficile* and a 14 mm zone when diluted. De Olanda et al. (2019) had different results of antimicrobial activity using *Salmonella typhimurium*, as it was resistant to *Tagetes minuta*. Furthermore, each organism showed unique inhibition zones for each essential oil, and this indicated that *Escherichia coli* (37 mm with undiluted *Pelargonium graveolens* and 5 mm with diluted *Pelargonium graveolens*) and *Staphylococcus aureus* (20 mm with undiluted

Pelargonium graveolens and 19 mm with diluted *Pelargonium graveolens*) had different mechanisms when they targeted the selected bacteria.

The results that are summarised in **Figure 4** and **Figure 6** show that *Pelargonium graveolens* essential oil demonstrated antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*. *Tagetes minuta* was previously studied by de Olanda et al. (2019), who discovered that it had a noteworthy antimicrobial effect on *Salmonella typhimurium* as it was more effective against this bacterium in this study (17 mm when undiluted and 13 mm when diluted) and *Clostridium difficile* (29 mm when undiluted and 14 mm when diluted) compared with other EOs (see **Figure 3** and **Figure 5**).

Furthermore, undiluted *Helichrysum splendidum* and *Mentha piperita* showed smaller inhibition zones against all the organisms compared to undiluted *Pelargonium graveolens* and *Tagetes minuta* (**Figure 2)** with the following inhibition zones: *Helichrysum splendidum* (*Staphylococcus aureus) = 10 mm; Salmonella typhimurium = 7 mm; Clostridium difficile = 13 mm*; *Escherichia coli = 19 mm; Mentha piperita (Staphylococcus aureus) = 14 mm; Salmonella typhimurium = 11 mm; Clostridium difficile = 16 mm*; and *Escherichia coli = 24 mm*. However, as undiluted essential oils *Pelargonium graveolens* and *Tagetes minuta* were more effective as they showed greater inhibition zones than the other two. Slightly different results were obtained when the oils were diluted because *Pelargonium graveolens* had an inhibition zone of 19 mm against *Staphylococcus aureus* and *Helichrysum splendidum* had a an inhibition zone of 18 mm against *Escherichia coli*. This means that *Helichrysum*

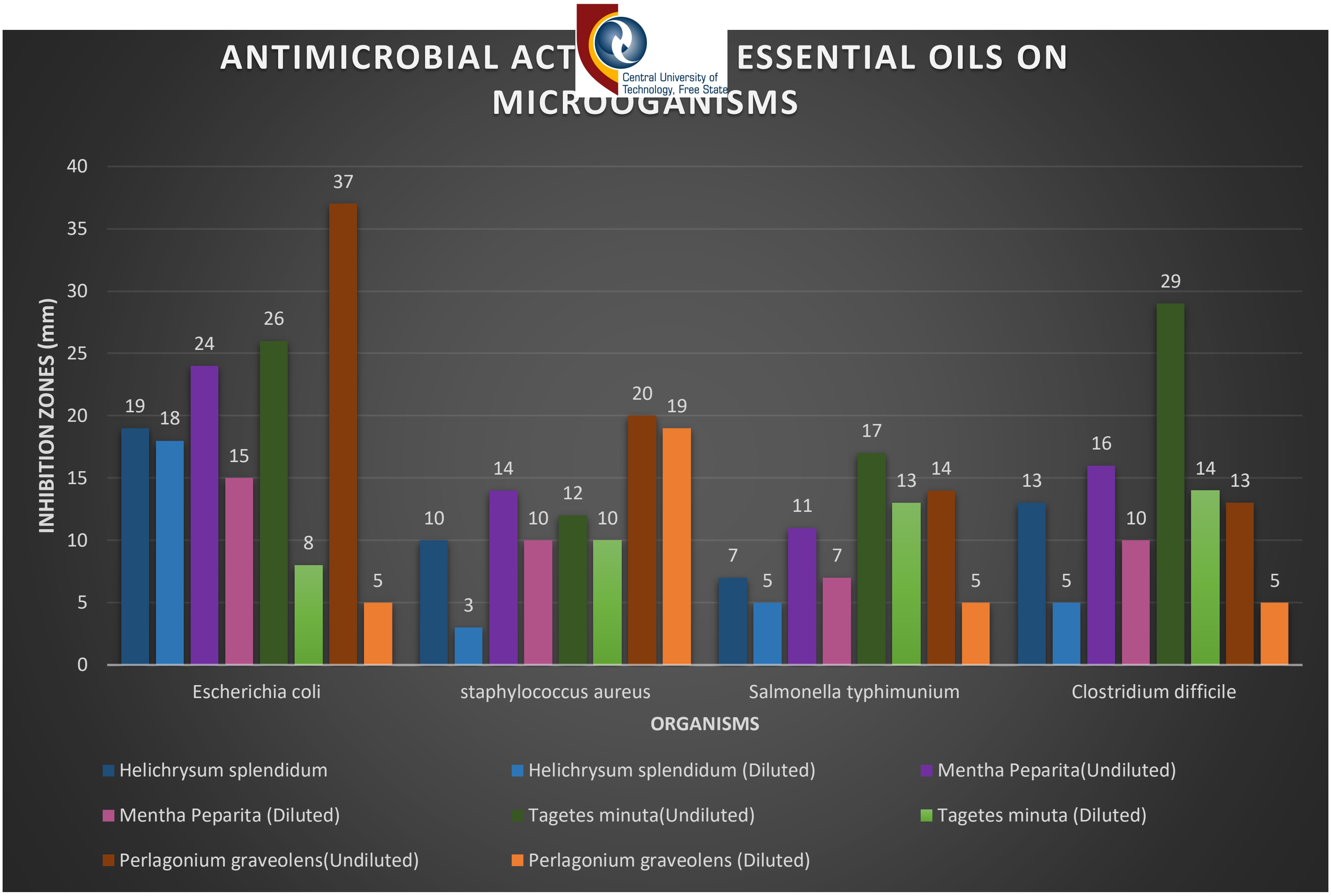

Figure 2*:* Antimicrobial activities of EOs (*Helichrysum splendidum, Mentha piperita, Tagetes minuta, Pelargonium graveolens*) against bacterial pathogens (*Escherichia coli, Staphylococcus aureus, Salmonella typhimurium, Clostridium difficile*) using bioassay preparation, measured in mm.

splendidum has potential when diluted although *Pelargonium graveolens* and *Tagetes minuta* also have potential when undiluted. However, this does not mean that *Helichrysum splendidum* and *Mentha piperita* did not have any effect on the organisms, but their antimicrobial activity was not as high compared to that of *Pelargonium graveolens* and *Tagetes minuta*. In addition to the tests on the essential oils against the selected organisms, ethanol was also tested against all the organisms, but it did not show any visible inhibition of growth. This indicated that ethanol had little or no effect on the tested organisms while the EOs produced noteworthy although varied results.

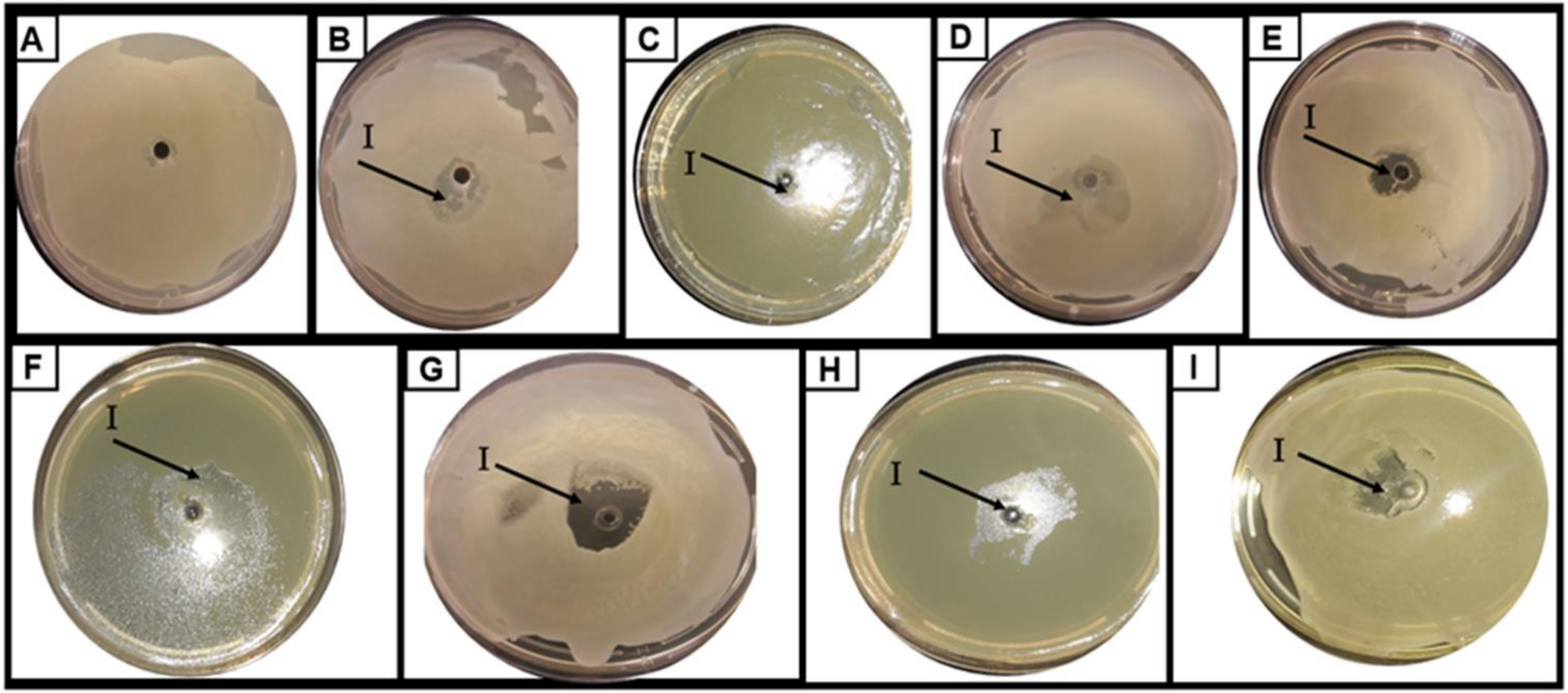

Figure 3: A bioassay showing cells of *C. difficile*: Untreated (control) cells (A), cells treated with *Helichrysum splendidum (B*= Undiluted oil, C= Diluted oil), *cells treated with Mentha piperita* (D= Undiluted oil, E= Diluted oil), cells treated with *Tagetes minuta* (F=Undiluted oil, G=Diluted oil), cells treated with *Pelargonium graveolens* (H= Undiluted oil, I= Diluted oil). I- indicates the inhibition zones of the treated cells

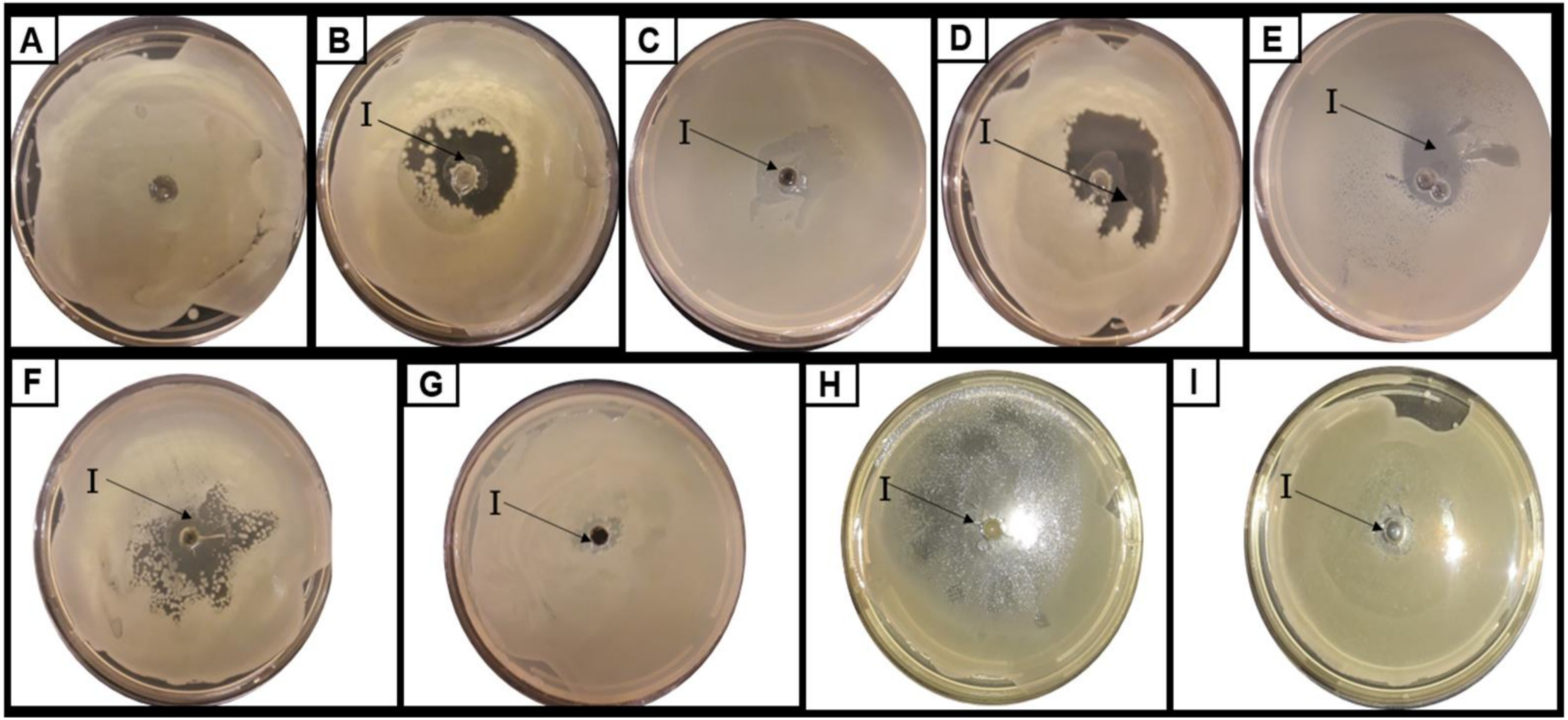

Figure 4: A bioassay showing cells *of E.coli*: Untreated(control) cells (A), cells treated with *Helichrysum splendidum* (B= Undiluted oil, C= Diluted oil), cells treated with *Mentha piperita* (D= Undiluted oil, E= Diluted oil), cells treated with *Tagetes minuta* (F=Undiluted oil, G=Diluted oil), cells treated with *Pelargonium graveolens* (H= Undiluted oil, I= Diluted oil). I- indicates the inhibition zones of the treated cells.

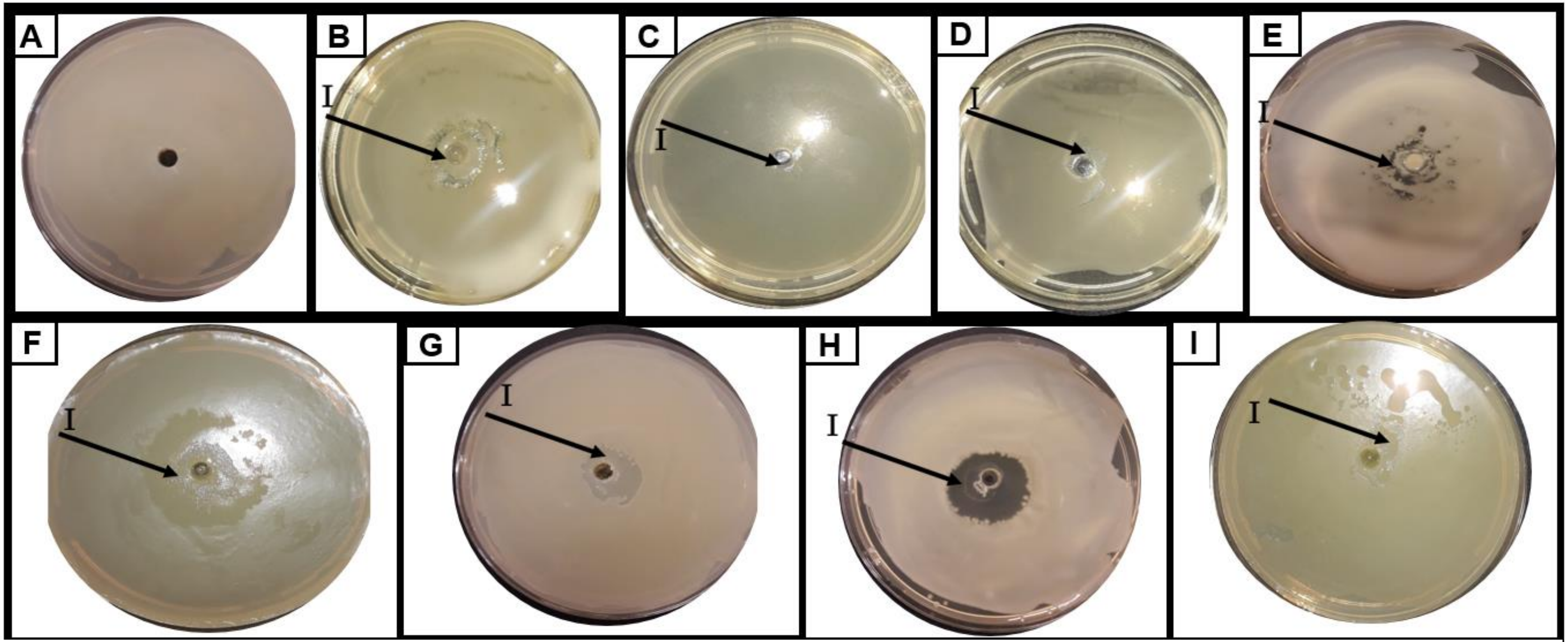

Figure 5: A bioassay showing cells of *S. typhimurium*: Untreated(control) cells (A), cells treated with *Helichrysum splendidum* (B= Undiluted oil, C= Diluted oil), cells treated wth *Mentha piperita* (D= Undiluted oil, E= Diluted oil), cells treated with *Tagetes minuta* (F=Undiluted oil, G=Diluted oil), cells treated with *Pelargonium graveolens* (H= Undiluted oil, I= Diluted oil). I- indicates the inhibition zones of the treated cells

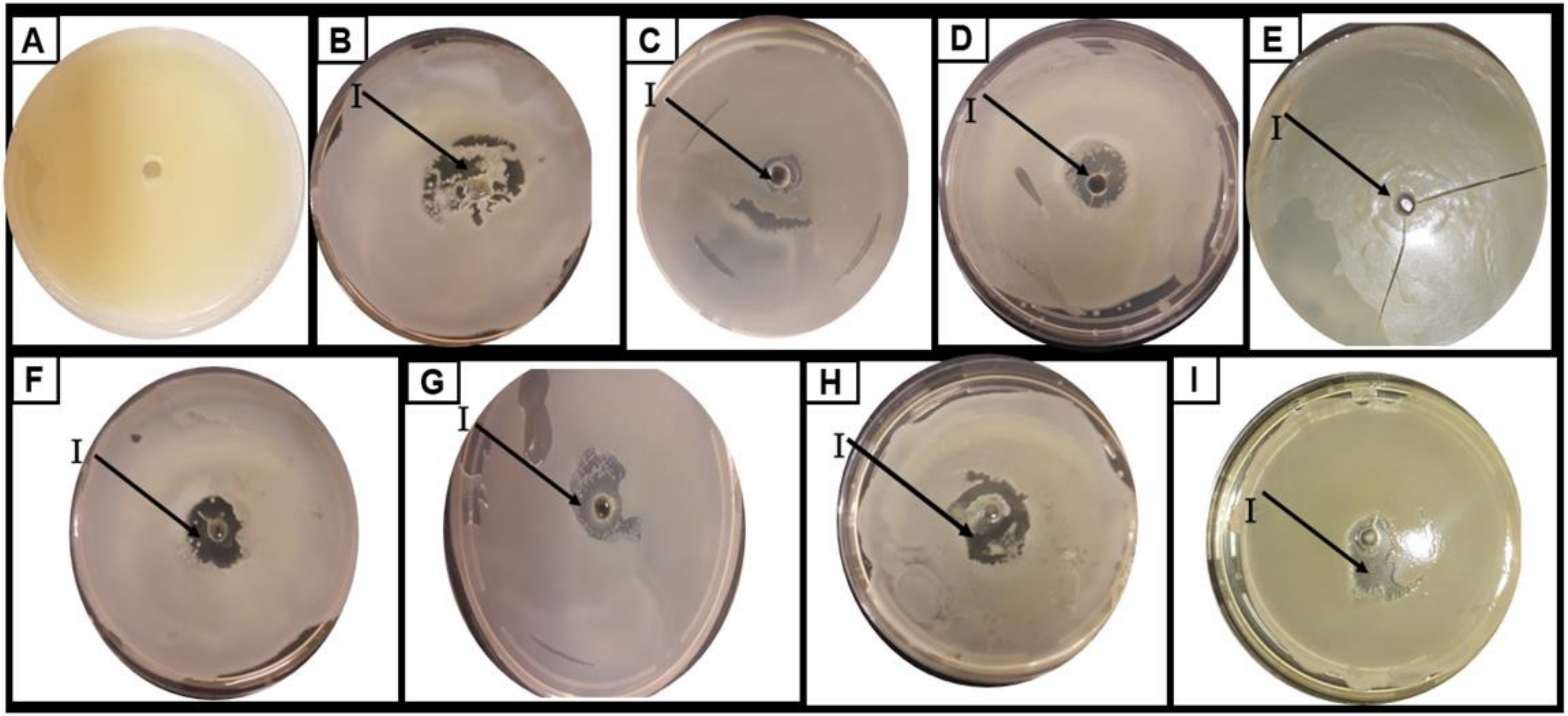

Figure 6: A bioassay showing cells of *S.aureus*: Untreated(control) cells (A), cells treated with *Helichrysum splendidum* (B= Undiluted oil, C= Diluted oil), cells treated wth *Mentha piperita* (D= Undiluted oil, E= Diluted oil), cells treated with *Tagetes minuta* (F=Undiluted oil, G=Diluted oil), cells treated with *Pelargonium graveolens* (H= Undiluted oil, I= Diluted oil). I- indicates the inhibition zones of the treated cells

3.4. Minimal Inhibitory Concentration (MIC)

The inhibitory effects of the essential oils were evaluated and the effects of different concentrations of each oil were observed for each organism. **Table 2** indicates that, for all four undiluted essential oils (*Helichrysum splendidum, Mentha piperita, Tagetes minuta,* and *Pelargonium graveolens*), there was no bacterial growth of *Clostridium difficile*. Moreover, using diluted *Tagetes minuta* showed a MIC of ≥12 µg/ml against this organism, which indicated that this EO was effective against this bacterium whether diluted or not. Diluted *Helichrysum splendidum* and *Mentha piperita* achieved an MIC of ≥50 µg/ml against *Clostridium difficile*. Furthermore, diluted *Pelargonium graveolens* achieved only minor growth at ≥100 µg/ml against *Clostridium difficile.* This indicated limited effectiveness of this oil on this organism because there was some growth even though it was minor. **Table 3** indicates that the essential oil that was most effective against *Salmonella typhimurium* was *Tagetes minuta* as it achieved an MIC of ≥25 µg/ml when diluted. When used undiluted no bacterial growth occurred. This was not the case with the other EOs. The second most effective essential diluted oil was *Pelargonium graveolens* with an MIC of ≥50 µg/ml against *Salmonella typhimurium*, while *Helichrysum splendidum* and *Mentha piperita* were the least effective essential oils against *Salmonella typhimurium* as they both showed an MIC of ≥100 µg/ml when they were diluted. However, *Pelargonium graveolens* showed greater antimicrobial activity against *Escherichia coli* with an MIC of ≥25 µg/ml when diluted and no bacterial growth occurred when undiluted (see **Table 4**). Both diluted *Tagetes minuta* and *Mentha Piperita* resulted in an MIC of ≥50 µg/ *Escherichia coli* and *Helichrysum splendidum* achieved an MIC of ≥100 µg/ml when diluted. When tested against *Staphylococcus aureus,* diluted *Pelargonium graveolens* achieved an

MIC of ≥50 µg/ml and when diluted no growth occurred (**Table 5**). Additionally, although diluted *Tagetes minuta* was not effective against three bacteria as there was no growth at certain concentration levels, but when used against *Staphylococcus aureus* minor growth (+) occurred at ≥100 µg/ml. *Mentha piperita* began to show an MIC of ≥100 µg/ml against this bacterium and *Helichrysum splendidum* also achieved medium growth at ≥100 µg/ml against this bacterium, which indicated that bacterial growth decreased but that the organism did not completely die.

Table 2: Minimal Inhibitory Concentration of EOs on *Clostridium difficile*

Dilution of EOs and ethanol(µg/ml)	≥100	≥50	≥25	≥12.5	≥6.3	≥3.1	≥1.6	≥0.8
Ethanol	+++	+++	+++	+++	+++	+++	+++	+++
Diluted *Pelargonium graveolens*	+	+	+	++	++	++	++	+++
Undiluted *Pelargonium graveolens*	-	-	-	-	-	-	-	-
Diluted *Helichrysum splendidum*	-	-	+	+	+	++	++	++
Undiluted *Helichrysum splendidum*	-	-	-	-	-	-	-	-
Diluted *Mentha piperita*	-	-	+	+	+	++	++	++
Undiluted *Mentha piperita*	-	-	-	-	-	-	-	-
Diluted *Tagetes minuta*	-	-	-	-	+	+	++	++
Undiluted *Tagetes minuta*	-	-	-	-	-	-	-	-

- Key **(-)** indicates no growth of bacteria, **(+)** indicates minor growth, **(++)** indicates medium growth, **(+++)** indicates strong growth. Colour GREEN indicates where the essential oil started to completely kill the organism and the colour BLUE indicates where the organism's growth started to decrease.

Table 3: Minimal Inhibitory Concentration of EOs on *Salmonella typhimurium*

Dilution of EOs and ethanol(μg/ml)	≥100	≥50	≥25	≥12.5	≥6.3	≥3.1	≥1.6	≥0.8
Ethanol	+++	+++	+++	+++	+++	+++	+++	+++
Diluted *Helichrysum splendidum*	+	++	++	++	++	+++	+++	+++
Undiluted *Helichrysum splendidum*	-	-	-	-	-	+	+	+
Diluted *Mentha piperita*	-	+	+	+	++	++	++	+++
Undiluted *Mentha piperita*	-	-	-	-	-	-	-	-
Diluted *Pelargonium graveolens*	-	-	+	+	+	++	++	+++
Undiluted *Pelargonium graveolens*	-	-	-	-	-	-	-	-
Diluted *Tagetes minuta*	-	-	-	+	+	+	++	++
Undiluted *Tagetes minuta*	-	-	-	-	-	-	-	-

- Key **(-)** indicates no growth of bacteria, **(+)** indicates minor growth, **(++)** indicates medium growth, **(+++)** indicates strong growth. Colour GREEN indicates where the essential oil started to completely kill the organism and the colour BLUE indicates where the organism's growth started to decrease.

Table 4: Minimal Inhibitory Concentration of EOs on *Escherichia coli*

Dilution of EOs and ethanol(µg/ml)	≥100	≥50	≥25	≥12.5	≥6.3	≥3.1	≥1.6	≥0.8
Ethanol	+++	+++	+++	+++	+++	+++	+++	+++
Diluted *Helichrysum splendidum*	-	+	+	+	+	+++	+++	+++
Undiluted *Helichrysum splendidum*	-	-	-	-	-	-	-	-
Diluted *Mentha piperita*	-	-	+	++	++	+++	+++	+++
Undiluted *Mentha piperita*	-	-	-	-	-	-	-	-
Diluted *Tagetes minuta*	-	-	+	+	+	+	++	++
Undiluted *Tagetes minuta*	-	-	-	-	-	-	-	-
Diluted *Pelargonium graveolens*	-	-	-	+	+	+	++	++
Undiluted *Pelargonium graveolens*	-	-	-	-	-	-	-	-

- Key **(-)** indicates no growth of bacteria, **(+)** indicates minor growth, **(++)** indicates medium growth, **(+++)** indicates strong growth. Colour GREEN indicates where the essential oil started to completely kill the organism.

Table 5: Minimal Inhibitory Concentration of EOs on *Staphylococcus aureus*

Dilution of EOs and ethanol(µg/ml)	≥100	≥50	≥25	≥12.5	≥6.3	≥3.1	≥1.6	≥0.8
Ethanol	+++	+++	+++	+++	+++	+++	+++	+++
Diluted *Helichrysum splendidum*	++	++	++	++	+++	+++	+++	+++
Undiluted *Helichrysum splendidum*	-	-	-	-	+	+	+	+
Diluted *Tagetes minuta*	+	+	+	+	++	++	++	++
Undiluted *Tagetes minuta*	-	-	-	-	-	-	-	-
Diluted *Mentha piperita*	-	+	+	++	++	++	+++	+++
Undiluted *Mentha piperita*	-	-	-	-	-	-	-	-
Diluted *Pelargonium graveolens*	-	-	+	+	+	+	++	++
Undiluted *Pelargonium graveolens*	-	-	-	-	-	-	-	-

- Key **(-)** indicates no growth of bacteria, **(+)** indicates minor growth, **(++)** indicates medium growth, **(+++)** indicates strong growth. Colour GREEN indicates where the essential oil started to completely kill the organism and the colour BLUE indicates where the organism's growth started to greatly decrease, and the colour GREY indicates where the organism's growth started to minimally decrease.

3.5. Morphological Changes Caused by Essential Oils on Bacterial Cells

SEM analyses were performed to detect and assess any morphological changes that occurred on the bacterial cells of the selected organisms after the application of selected essential oils. The results revealed that *Tagetes minuta* was able to cause structural changes to the bacterial cells of *Clostridium difficile* and *Salmonella typhimurium* (**Figure 7** and **Figure 8**). These changes are visible as holes on the cell wall and this must have compromised the cell integrity by creating an imbalance of electrolytes that triggered swelling of the cell, incomplete cell division, and eventually cell death. Das et al. (2022) discussed similar findings, arguing that essential oils are able to disturb the motion of normal microbial growth by reducing the ATP production and modifying protein synthesis.

Moreover, these structural modifications suggest energy limitations and therefore the inhibition of the toxin production by the bacterial cells, as proposed by Dashamiri et al. (2019) and Zhu et al. (2022). It is possible that the secretion of toxins was prevented by these modifications in the bacterial membrane due to the attachment of the essential oil that disturbed the phospholipid bilayer and led to the trans-membrane transport process, as proposed by Zhu et al. (2022). This may therefore have limited the release of toxins into the immediate environment.

Moreover, *Pelargonium graveolens* managed to cause some structural changes to the cells of *Escherichia coli* and *Staphylococcus aureus* (see **Figure 9** and **Figure 10**). The changes that were observed in the cell membranes suggest that *Pelargonium graveolens* caused incomplete cell division resulting in cell cycle progression being constrained, as was found by Dashamiri et al. (2019) and Das et al. (2022). The latter authors also observed rupturing of the cell membrane, the deformation of the cell, and roughness on the cell wall. **Figure 9** indicates that the death of the *Escherichia coli* bacterial cell was caused by *Pelargonium graveolens*. Cell death occurred as the cell membrane ruptured and this could have led to the inhibition of electron transportation for energy production, disruption of the proton motive force, protein translocation, and synthesis of cellular components. This finding suggests the effectiveness of *Pelargonium graveolens* in combating *Escherichia coli.*

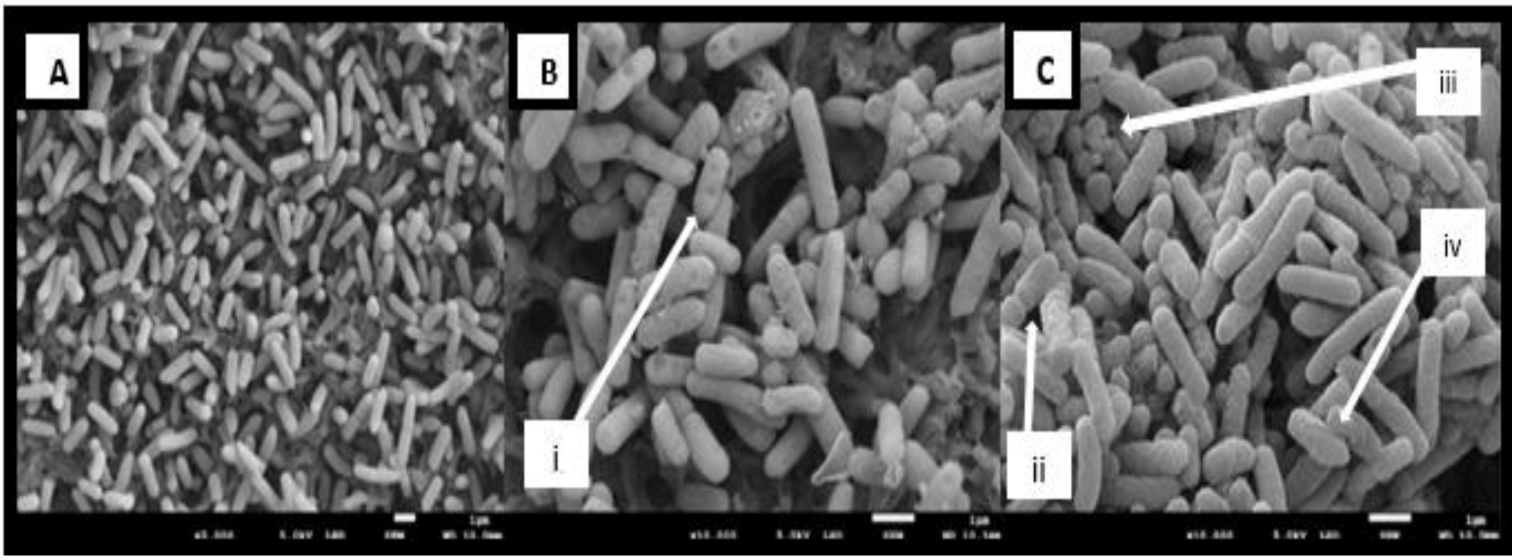

Figure 7*:* Scanning Electron Microscopy analysis of *Clostridium difficile*. A= Untreated bacterial cells, B and C= Treated cells *Tagetes minuta*. i= Holes on the surface of the cell wall, ii= Incomplete cell division, iii=loss of contents, iv=shape of the cell changed and there is swelling of the cell.

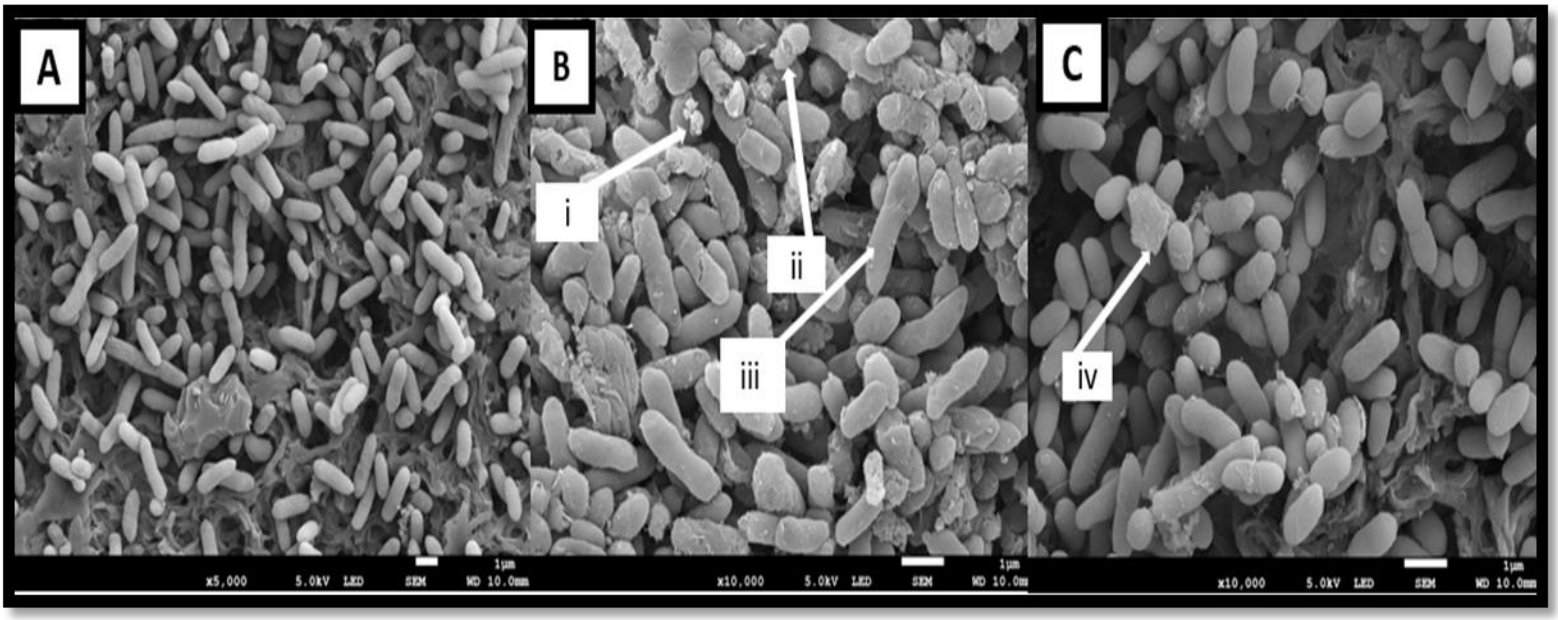

Figure 8*:* Scanning Electron Microscopy of *Salmonella typhimurium*. A= Untreated cells, B and C= Cells treated with *Tagetes Minuta*. i= loss of contents, ii= hole, iii= Deformation and swelling of the cell, iv= abnormal cell rupturing.

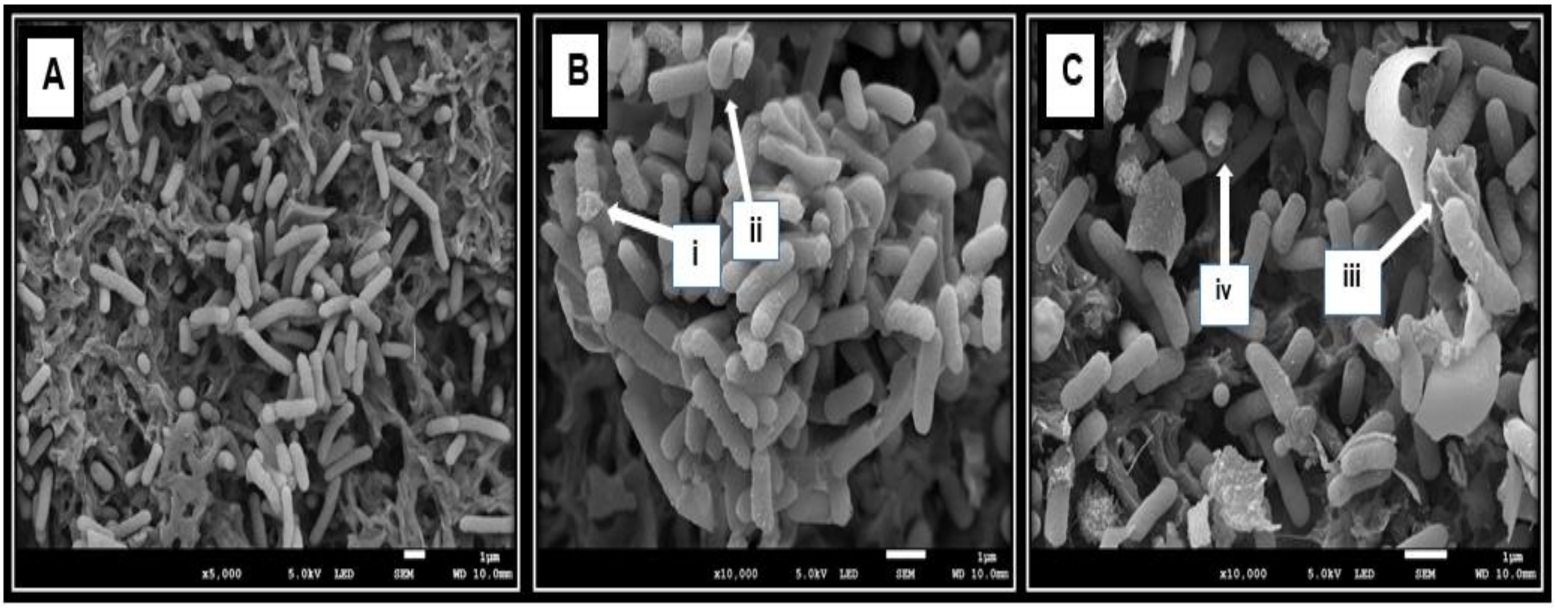

Figure 9: Scanning Electron Microscopy of *Escherichia Coli.* A= Control cells, B and C= Bacterial cells treated with *Pelargonium graveolens*. i= Rough cell surface/disruption of cell membrane, ii=incomplete cell division, iii=complete cell death, iv=holes formed on the cell wall.

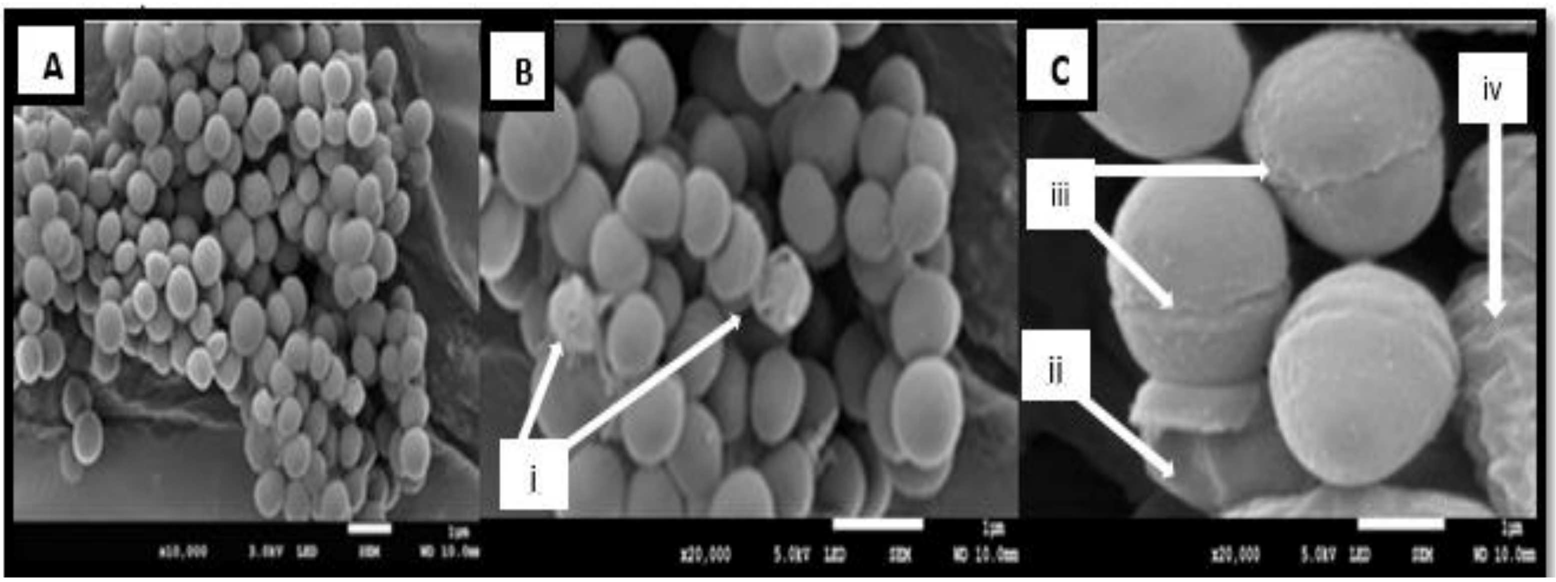

Figure 10: Scanning Electron Microscopy of *Staphylococcus aureus*. A= Untreated cells, B and C= Cells treated with *Pelargonium graveolens*. I and ii= loss of contents and formation of holes, iii= incomplete cell division, iv= rough surface of the cell wall.

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CHAPTER 4: CONCLUSION AND FUTURE RESEARCH

4.1. INTRODUCTION

Infections that cause diarrhoea and that result in the death of about 500 000 people per annum worldwide are considered an important health problem by WHO (World Health Organization, 2005; Liu et al., 2016). Such infections and severe diarrhoea associated with them are also responsible for the deaths of more than 90% of children under the age of five years in low- and middle-income countries (Demissie et al., 2021). In South Asia and sub-Saharan Africa, unresolved diarrhoea accounts for 88% of deaths in young children (Demissie et al., 2021).

Several factors play a role in causing diarrhoea in humans and especially in children under the age of five (Demissie et al., 2021). These factors include lack of maternal education, contaminated sources of drinking water, low socio-economic status, poor breastfeeding practices, inappropriate disposal of stools and faeces, family size, maternal employment status, maternal age, limited hand washing facilities and poor handwashing practices, and the number of children in the household (Demissie et al., 2021). For example, many mothers of young children are inexperienced and do not know how to practise safe hygiene to avoid infecting a child (Demissie et al., 2021). A large number of children in a household is also a factor that escalates the chances of contamination and infection transmission from one child to another (Najim et al., 2019). As infections that cause diarrhoea can be life-threatening, such illnesses demand attention regarding effective treatment and control (Najim et al., 2019). Kayambankadzanja et al. (2020) stated that antibiotics that were developed to control organisms associated with diarrhoea were at first effective and reduced several diarrhoeal cases in the paediatrics field. Najim et al. (2019) and Mantegazza et al. (2018) agree. However, bacterial organisms developed resistance to antibiotics, which has caused severe problems in the medical field as many such infections have become very difficult to cure. Researchers have thus begun to search for alternatives to antibiotics, which also have side-effects (Gibson et al., 2015; Chandra et al., 2017). It has been argued that antibiotics are overused and that this has resulted in a substantial increase in the rates of antibiotic resistance to bacterial strains (Serwecińska, 2020). Consequently, the United Nations recognises resistance to antibiotics as a global health threat that requires urgent attention (Kayambankadzanja et al., 2020). Alternatives in the treatment of microbial infections have been in demand and natural antimicrobials such as essential oils have been studied and used to great effect (Nazzaro et al., 2013; Bouyahya et al., 2019). The use of essential oils has shown positive results and they are regarded as a promising medicament in the pharmaceutical industry (Nazzaro et al., 2013; Chandra et al., 2017). In light of the foregoing, the aim of this study was to investigate the activity of essential oils against selected pathogens that are known to be foodborne and that cause infections leading to diarrhoea. The objectives were to:

- Characterise four essential oils, namely *Tagetes minuta, Mentha piperita, Helichrysum splendidum, and Pelargonium graveolens* using gas chromatography-mass spectrometry (GC-MS);
- Determine the minimum inhibitory concentrations (MIC) of the selected essential oils, and to evaluate their inhibitory effect on *Clostridium difficile*, *Escherichia coli*, *Salmonella*, and *Staphylococcus aureus* using the bioassay method; and
- Determine the effect of the essential oils on the cell morphology of the selected bacteria using scanning electron microscopy (SEM).

4.2. DISCUSSION

4.2.1 Gas chromatography and mass spectrometry

The results were presented in Chapter 3. The composition of the selected essential oils was evaluated and analysed using gas chromatography coupled with mass spectrometry. The compounds that were identified in the essential oils and used in this study were mostly terpenes that are known to possess antimicrobial properties (Omayma et al., 2020). For instance, it was determined that *Helichrysum splendidum* had five main compounds, namely p-Cymene (5.44%), Eucalyptol (1,8-Cineole) (15.9%), α-Pinene (6.28%), α-Phellandrene (5.63%), and β-Pinene (36.76%). The same compounds were observed by Mashigo et al. (2015).

When analysed, *Helichrysum splendidum* demonstrated persistent antimicrobial activity on the organisms that were used, although its activities were limited compared to those of *Tagetes minuta* and *Pelargonium graveolens*. Ejaz et al. (2020) found similar results, and argue that the activity of *Helichrysum splendidum* tends to vary due to environmental differences. Furthermore, the main compounds in *Mentha piperita* were determined as 1,8-cineole (eucalyptol) menthofuran (12.57%), menthol (33.05%), menthone (24.02%), and menthyl acetate (8.90%), but these showed minor to medium antimicrobial activity on the investigated organisms. *Tagetes minuta,* that had shown antimicrobial activity in a previous study (de Olanda et al., 2019), had four main constituents, namely p-Cymene, 1,8-cineole (eucalyptol) (3.30%), 2-Bornanone (camphor) (1.15%), trans-Thujone (β-thujone) (14.45%), and α-Thujene (71.59%). *Pelargonium graveolens* had the following compounds: Citronellyl acetate (19.45%), Citronellol (24.28%), Geraniol (17.83%), and 2-Phenylethanol (12.54%). These components were also indicated by Okla et al. (2022). Geraniol and citronellol are active compounds that inhibit the growth of microbes such as *Escherichia coli* and *Salmonella typhimurium* by causing damage to cellular membrane integrity or function (Okla et al., 2022).

Although *Pelargonium graveolens* showed great potential and was identified as the most promising essential oil to use against *Escherichia coli* among those that were tested, this does not mean that the other three tested oils were not effective, as their effectiveness was just lower than that of *Pelargonium graveolens* (**Figure 4)**. In a study by Chebbac et al. (2021), *Pelargonium graveolens* showed an inhibition zone of 57 mm against *Escherichia coli* and 37 mm against *Staphylococcus aureus.* When these effects are compared to those of the current study, it suggests that the effect of essential oils' bacterial inhibition capacity differ depending on the concentration of constituents present in them. Moreover, because the inhibition zone in the previous study was as greater than that of the current study, it is argued that *Pelargonium graveolens* does indeed have the potential to successfully inhibit growth in microorganisms. For instance, based on bioassay testing, *Pelargonium graveolens* showed the best results with the best inhibition diameter zone of 37 mm diameter against *Escherichia coli* (**Figure 4**)*.* Therefore, although previous studies (Nazzaro et al., 2013; Silva et al., 2017) suggest that Gram-negative bacteria are more likely to be resistant to essential oils than Gram-positive bacteria, however, this study and a previous study by Chebbac et al. (2021), demonstrated that *Escherichia coli* is susceptible to *Pelargonium graveolens*.

Furthermore, when the minimum inhibitory concentration (MIC) was observed for all the essential oils, it was noted that they could all inhibit the growth of the selected organisms when they were not diluted with ethanol. This was evident in the observation that there was no bacterial growth when undiluted EOs were applied. However, diluted *Tagetes minuta* (**Table 2)** had the best MIC of ≥12 µg/ml against *Clostridium difficile*, which indicated that this oil was more effective against this bacterium than the other three essential oils used (*Helichrysum splendidum*, *Pelargonium graveolens*, and *Mentha piperita*). **Table 3** indicates that the essential oil that was most effective against *Salmonella typhimurium,* with an MIC of ≥25 µg/ml, was *Tagetes minuta.* Essentially, when undiluted the application of this oil resulted in no bacterial growth and, when diluted, it had an MIC of ≥25 µg/ml, which was a better result than any of the other essential oils achieved.

Furthermore, *Pelargonium graveolens* showed the best antimicrobial activity against *Escherichia coli* (with an MIC of ≥25µg/ml when diluted and no bacterial growth when undiluted - **Table 4**) and against *Staphylococcus aureus* (with an MIC of ≥50 µg/ml when diluted and no bacterial growth when undiluted - **Table 5**).

4.2.2 Scanning electron microscopy (SEM)

Methodologically, a further investigation was conducted to determine if any morphological changes occurred in the cell structure of the selected bacteria (*Clostridium difficile*, *Escherichia coli*, *Salmonella typhimurium*, and *Staphylococcus* *aureus*). The results revealed that the essential oils were indeed able to affect the cell structure of these organisms negatively, which was the desired effect. To determine the morphological changes in each organism, scanning electron microscopy (SEM) was performed and, holistically, the results showed the following changes in the cells of the organisms (**Figure 7, Figure 8, Figure 9*Figure 9*,** and **Figure 10**):

- The cell walls showed roughness;
- Holes had formed on the cell walls;
- Cells were swollen;
- Incomplete cell division;
- Loss of cellular content; and
- Cell death.

However, the four essential oils (*Helichrysum splendidum*, *Pelargonium graveolens*, and *Mentha piperita* and *Tagetes Minuta*) affected the bacterial strains differently. For instance, *Tagetes Minuta* (**Figure 7** and **Figure 8**) caused holes on the cell wall, swelling of the cell, and incomplete cell division resulting in cell cycle progression being constrained in *Clostridium difficile* and *Salmonella typhimurium*, while *Pelargonium graveolens* caused deformation of the cells and roughness on the cell walls of *Escherichia coli* and *Staphylococcus aureus* (**Figure 9** and **Figure 10**).

It was concluded that, because all the essential oils affected the cell walls of the organisms as demonstrated by the SEM results, their ability to insert into the phospholipids bilayer of the cell wall because of their richness in phenolic compounds led to a trans-membrane transport process that limited the release of bacterial toxins into the immediate environment (see Chapter 3). Cell death also occurred as a result of the disruption of the cell membrane. This rupturing must have led to the inhibition of electron transportation for energy production, disruption of the proton motive force, protein translocation, and synthesis of cellular components.

4.3. LIMITATIONS OF THE STUDY

- The study was delimited to **Gas chromatography** and **mass spectrometry** as well as **electron microscopy** investigations.
- The bacterial samples were obtained from a reputable supplier in South Africa and, for health and safety purposes during the COVID-19 period (February 2020 to June 2022), no samples were collected in the field. It was therefore not verified if the bacterial samples were foodborne-derived, but it was assumed that this was a possibility as the selected bacteria notoriously cause foodborne infections.
- An extensive literature review was conducted as a backdrop to the laboratory investigations. This literature investigation adequately explored the plight of young children who contract infections that cause diarrhoea. No bacteria were therefore directly obtained from faeces or the gut for analysis in this study.

4.4. CONCLUSION AND RECOMMENDATIONS

This study confirmed earlier findings as it adequately demonstrated that essential oils can be used as alternatives to antibiotics to inhibit bacterial growth. *Tagetes minuta* and *Pelargonium graveolens* successfully inhibited the growth of selected bacterial organisms and disrupted their morphology (**Chapter 3**). The use of essential oils in the medical field is a growing industry that is bringing change to pharmaceutical medications. They are indeed a promising alternative to antibiotics. However, the formulation and application of essential oils still need extensive investigation to determine how they can best be used to treat human infections caused by bacteria.

Although no data were obtained and utilised to explore the effects of bacterial infections and the use of essential oils in children to curb the occurrence of diarrhoea, the literature review confirmed the extensive impact of bacterial infections on humans, and especially on young children. Based on the findings, the following recommendations are thus offered to address the threat of bacterial infections that may cause untimely death:

4.4.1 Recommendations

- There is a fallow field for essential oil studies that should explore how they can be used on surfaces and equipment to ensure a safe environment for humans, but especially for children who are highly susceptible to bacterial infections, as was indicated by the literature review.
- The education of women to enhance their knowledge and improve their practice of basic preventive measures – such as proper hand hygiene using disinfectants

that contain natural substances with no side effects - should be introduced, especially in areas where their education has been marginalised.

- A review of the contents and quality of the school and basic adult education curricula to include information about natural health remedies seems necessary, which is a notion that is supported by various authors such as Demissie et al. (2021).
- The use of essential oils as an alternative to treating diarrhoea to avoid its reoccurrence should be encouraged among and by medical practitioners and care givers in the medical and paediatric fields.
- The South African population still needs to practise safe hygiene regardless of the abolishment of COVID-19 protocols and practices, because poor hygiene is the main culprit that causes infection-induced diarrhoea, especially in children.
- The use of natural medicines should not be restricted to the so-called ‘alternative health care field’ or traditional healing practices, but should be endemic to all medical treatments in clinics and hospitals alike. Such medications will have fewer (or no) side effects in humans and can be applied across a wide spectrum of treatment protocols.

4.5. FUTURE RESEARCH

This study suggests that the following research areas need to be considered:

- Cytotoxicity to determine whether the essential oils that are used are safe for human consumption.
- Antimicrobial activity of antibiotics on organisms to determine the different morphological changes on organisms when compared to essential oil activity.
- A comparison between surface disinfectants that contain alcohol and essential oils to determine whether alcohol-based disinfectants are as effective as essential oil-based disinfectants. This may lead to the development of essential oil-based disinfectants.

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