ANTIMICROBIAL ACTIVITY OF ESSENTIAL OILS AS LIQUID AND VAPOUR APPLICATIONS

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A thesis submitted in partial fulfilment of the requirements of the University of the West of England, Bristol for the degree of Doctor of Philosophy (PhD)

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December 2019

Word Count: 32,613

ABSTRACT

Essential oils produced by plants have been used as antimicrobial agents historically, however, many essential oils remain under-exploited as alternatives to current antimicrobials. Therefore, the aim of this thesis was to evaluate the antibacterial efficacy of selected essential oils against bacteria which have the ability to form biofilms. The broad-spectrum activity of several essential oils is demonstrated, with subsequent focus on the strong antimicrobial activity of cinnamon essential oil extracted from *Cinnamomum zeylanicum* bark. Cinnamon bark essential oil showed broad spectrum activity against a range of bacteria, some of which are relevant clinically, including *Pseudomonas aeruginosa* which is capable of forming biofilms and implicated in many human diseases. Cinnamon bark essential oil killed planktonic *P. aeruginosa* at concentrations as low as 0.125% (v/v), and within 2 min when at concentrations of ≥1% (v/v). It also exhibited anti-biofilm activity, in both liquid and vapour form, against *P. aeruginosa* biofilms grown using a Centre for Disease Control biofilm reactor on polycarbonate and stainless steel surfaces. Liquid application of 2% (v/v) resulted in biofilm eradication in as little as 10 min. When assessed in a novel testing chamber, designed and built as part of this project, cinnamon EO reduced biofilms at vapour concentrations as low as 0.2 µL cm^{-3}. These results provide clear evidence for the potential of cinnamon bark essential to be considered as a novel antimicrobial, and to contribute to the improvement of hygiene and sanitation. As well as addressing the global issue of antibiotic resistance, data presented also provide evidence for the ability of essential oils to inhibit and eradicate biofilms, which increases tolerance of pathogenic bacteria to antimicrobials. Data presented here provide the basis of additional work to investigate development of disinfectant products, evaluate essential oil toxicity, and further study the role of essential oils in combatting antimicrobial resistance.

ACKNOWLEDGEMENTS

I would first like to thank my supervisors, Emmanuel Adukwu and Peter Spencer-Phillips, for their roles in this project, their guidance, and for helping me to become the researcher I am. I would also like to thank the co-funders of this PhD, Medical Wire and Equipment, without whom this PhD would not have been possible.

I'd like to thank all the staff at UWE. A special thanks to the micro technicians, Lee, Gill and Barbara, for their constant support, admirable patience and putting up with my singing through thin walls. A big thanks to Dave Patton for his SEM skills, to Paul Bowdler for his help with GC-MS, to Dave Corry and the rest of the technical team for facilitating such a great workspace to carry out research, and to Paul from stores for always having what I need and being my chief cardboard supplier.

A big thank you to all of my friends, colleagues, academics and PGRs in the department. I thank them for the advice they've offered, drinks they've bought, bake off sweepstakes, scavenger hunts, annual BBQs, Christmas quizzes, and endless innuendoes.

Llama and Lizzy, you've been there in the good times and the bad, and for that I am forever grateful. Thank you for letting TMI no longer be in our vocabulary, for pyjamas and slippers being a given, and for always keeping me in check with pragmatic perspectives. I don't think I would have made it this far without you. I love you guys, and I will cherish our friendship always.

Malwyn, you've been so supportive, thoughtful and just the best human being. Thank you for keeping me sane and putting up with me during this emotional rollercoaster. You've kept me happy and smiling, I love you.

To my family, thank you for your strength and encouragement. You constantly remind me to stay true to myself and strive for the top. I wouldn't be me without you, and for that I am grateful.

And to Coco, my beautiful sister. Never a day goes by when you aren't missed. You'll never know the impact you've made and how you've shaped me into the person I am. You've taught me to be strong, to be brave in my beliefs, to love fiercely, and live with no regrets.

I dedicate this work to you.

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degrees Celsius
µL	Microlitres
µm	Micrometre
AHL	Acyl-homoserine Lactone
AI-2	Autoinducer 2
AMR	Antimicrobial Resistance
ANOVA	Analysis of Variance
AST	Antimicrobial Susceptibility Testing
ATCC	American Type Culture Collection
BS EN	British Standards European Norm
BSA	Bovine Serum Albumin
CBR	CDC Biofilm Reactor
CDC	Centre for Disease Control
CFU	Colony Forming Units
CFU mL^{-1}	Colony Forming Units per Millilitre
CLSI	Clinical and Laboratory Standards Institute
CV	Crystal Violet
DMS	Dimethyl Sulfide
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribose Nucleic Acid
EO	Essential Oil
EPA	Environmental Protection Agency
EPS	Exopolysaccharide
EU	European Union
EUCAST	European Committee for Antimicrobial Susceptibility Testing
FDA	Food and Drug Administration
g L^{-1}	Gram per Litre
GC-MS	Gas Chromatography Mass Spectrometry
GRAS	Generally Regarded/Recognised as Safe
h	Hour
HC	High Concentration
HMDS	Hexamethyldisilizane
HPV	Hydrogen Peroxide Vapour
LC	Low Concentration

log	Logarithmic
m/z	Mass to Charge Ratio
MBC	Minimum Bactericidal Concentration
MBEC	Minimum Biofilm Eradication Concentration
MBIC	Minimum Biofilm Inhibition Concentration
MDR	Multidrug Resistance
MHA	Mueller Hinton Agar
MIC	Minimum Inhibitory Concentration
min	Minute
mL	Millilitre
mmHg	Millimetre of Mercury
MRSA	Methicillin Resistant *Staphylococcus aureus*
MSSA	Methicillin Susceptible *Staphylococcus aureus*
NA	Nutrient Agar
NCTC	National Collection of Type Cultures
nm	Nanometre
OD	Optical Density
P	Probability
PBS	Phosphate Buffered Saline
PC	Polycarbonate
QS	Quorum Sensing
SEM	Scanning Electron Microscopy
SIFT-MS	Select Ion Flow Tube Mass Spectrometry
SPME	Solid Phase Microextraction
SS	Stainless Steel
TSA	Tryptone Soy Agar
TSB	Tryptone Soy Broth
TSC-1	Tolerance Subculture 1
TSC-2	Tolerance Subculture 2
UHMW	Ultra-High Molecular Weight
v/v	Volume per Volume
VOC	Volatile Organic Compounds
w:w	Weight to Weight
WEDD	Wet Effluent Diffusion Denuder

CHAPTER 1

1 INTRODUCTION

1.1 Antimicrobial resistance (AMR)

1.1.1 Origin and impact

In 1929, Sir Alexander Fleming demonstrated the potential antimicrobial use of penicillin. In 1943, penicillin was introduced for therapeutic use (Ventola 2015) and by the late 1940s, many antibiotics became commercially available, becoming crucial in combating previously untreatable infections and diseases (Debabov 2013). However, Fleming proposed that inappropriate use of penicillin might cause *Staphylococcus aureus* to mutate, leading to more severe infections and the transmission of these resistant strains from host to host, thus predicting AMR (Rosenblatt-Farrell 2009). Even before the introduction of penicillin in 1943, in 1940, resistance to this drug was already demonstrated (Ventola 2015). Thus, in agreement with the evidence, and true to Fleming's warnings, within one year of widespread use, substantial numbers of staphylococcal strains were exhibiting resistance to penicillin (Lobanovska and Pilla 2017). A few years later, over 50% of *S. aureus* species were no longer susceptible (Alanis 2005).

Approximately 100,000 tons of antibiotics are produced globally per year (Martens and Demain 2017), and for every 10 min passed, 2 tonnes of antibiotics are used (Harbarth *et al.* 2015). Not only have bacteria developed resistance but many strains have become resistant to multiple antibiotics and chemotherapeutic agents, termed multi-drug resistance (MDR; Nikaido 2009). MDR can be defined as non-susceptibility to at least one agent, in three or more antimicrobial categories (Magiorakos *et al.* 2012). The Centre for

Disease Control and Prevention (CDC) categorises MDR bacteria into one of three threat levels: urgent, serious or concerning (Figure 1.1; CDC 2018).

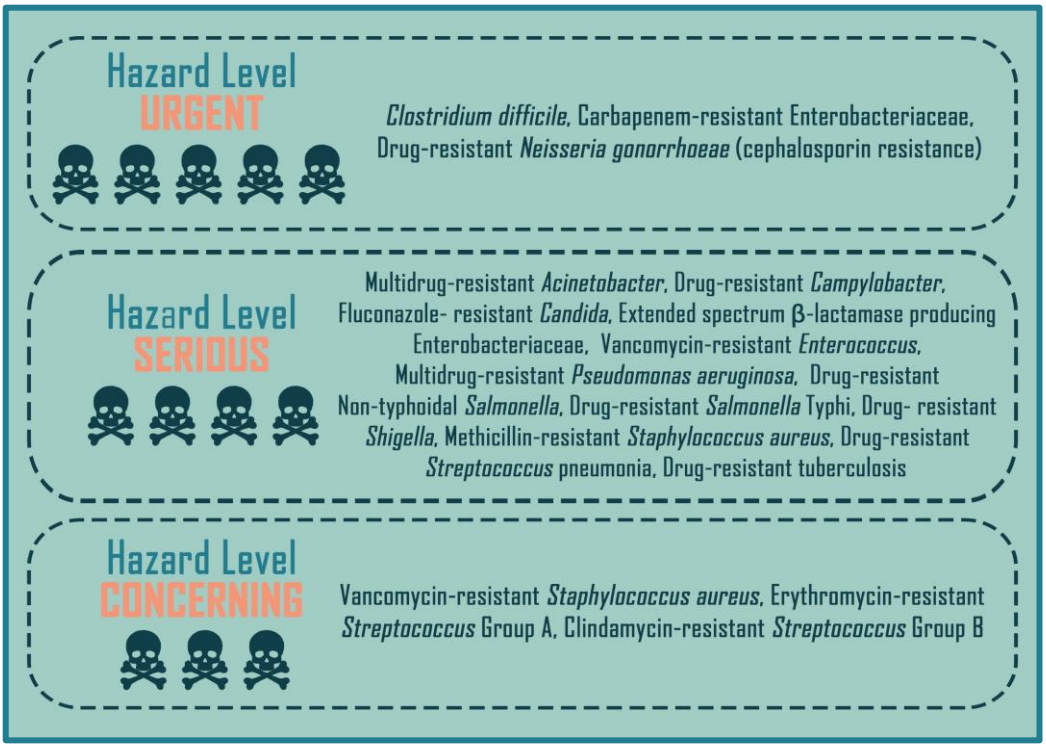


Figure 1.1 – Organism Threat Levels.

Classification of organisms by threat level as outlined by the Centre of Disease Control (2018). Image created by author using data from CDC (2018).

Of these organisms, the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) are reported to be the leading cause of nosocomial infections throughout the world (Santajit and Indrawattana 2016). The global threat of MDR bacteria has raised the need for urgent therapeutic discoveries, improvement of existing infection control, and development of antimicrobial practices. Microorganisms, more than ever before, are able to combat antimicrobial drugs, leading to unsuccessful management, perseverance and infection spread (Tanwar *et al.*

2014). Within the EU, MDR infections are responsible for approximately 25,000 patient deaths per year, with over 63,000 patient deaths per year in the United States caused by hospital-acquired bacterial infections (Aminov 2010). MDR bacterial infections result in extra healthcare costs and productivity losses costing the EU at least 1.5 billion euros each year (Department of Health 2016). Deaths attributable to AMR are expected to reach 10 million per year by 2050, which would overtake cancer as the leading cause of death (O'Neill 2014).

1.1.2 Drivers of resistance

One of the biggest drivers of AMR is the overuse and abuse of antibiotics. Their abundant consumption since introduction and inappropriate distribution has played a massive role in AMR (Ventola 2015). The substantial use of antibiotics in agriculture and aquaculture, for growth promotion and reduction of disease, (Prestinaci *et al.* 2015), and the lack of funding and budgets within healthcare for new effective antibiotics to be available commercially (Levy and Bonnie 2004), also contribute to AMR. However, though antibiotic misuse is a primary driver for AMR, it is not the only culprit. Biocides, metals and naturally occurring resistance genetics all play a role in increased AMR (Singer *et al.* 2016). Biocides such as ethanol, formaldehyde, chlorhexidine,

triclosan, and quaternary ammonium compounds, and metals which originate from drainage water, household effluent and traffic related emissions both contribute to the co-selection of genes that promote resistance (Singer *et al.* 2016). Resistance transmission routes are distributed throughout our

everyday life (Figure 1.2) and it is important to address each root cause in order to combat AMR.

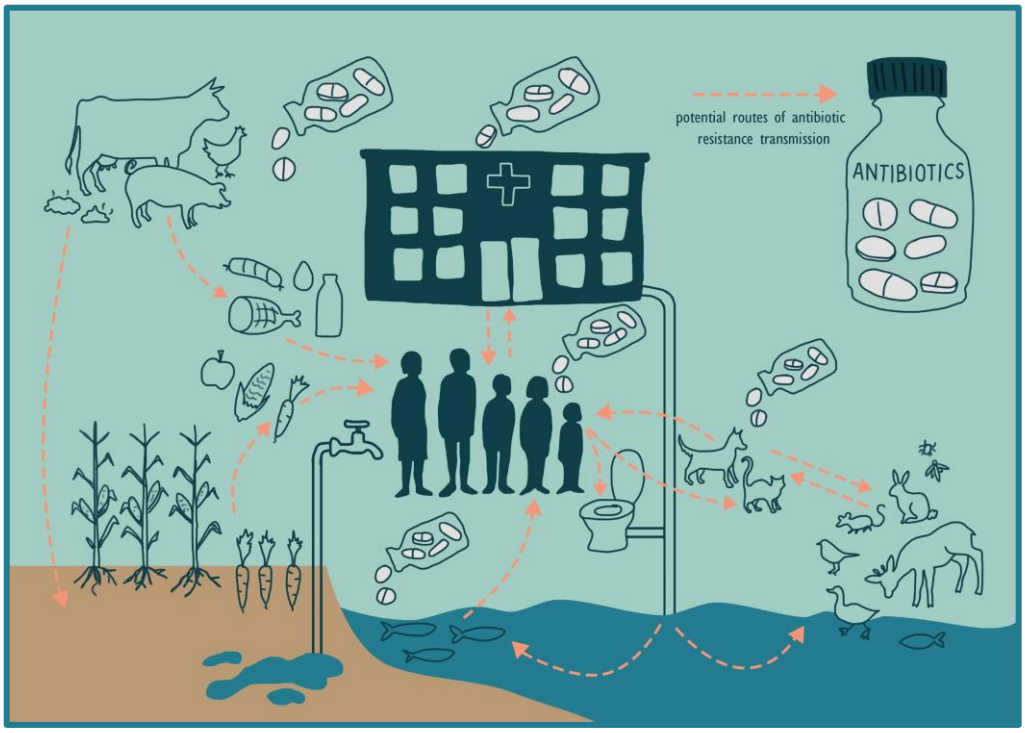


Figure 1.2 – Potential routes of transmission of resistant bacteria. Antibiotics used in agriculture and farming transmit resistant bacteria to both humans and aquatics via food and water run-off. Waste water systems transmit to aquatics and wildlife, which in turn may transmit to domestic animals and humans. Bacteria which are resistant to antibiotics used in hospital environments transmit to the population. (Image reproduced from Harbarth *et al.* 2015; CC BY 4.0)

1.1.2.1 Inappropriate prescribing and misuse

Amongst the European countries, the UK is in the lower half with regards to outpatient antibiotic use (Smieszek *et al.* 2018). Despite this, it is estimated that 20% of antibiotics prescribed are done so unnecessarily (Courtenay *et al.* 2019). Problems arise when doctors have an inaccurate diagnosis and prescribe antibiotics as a precaution, or a broad spectrum drug is used. A

recent report by Public Health England revealed that the majority of prescriptions given for antibiotics were for respiratory or urinary tract infections, although nearly a third of these gave no clinical reasoning (Public Health England 2018). Results of the report revealed the staggering differences between drugs being prescribed versus the ideal number of cases where prescription is necessary (Table 1.1), thus highlighting the opportunity for practices across the country to reduce their prescribing rates.

Table 1.1 – Prescription Rates.

Public Health England (2018) evaluation of rates of antibiotic prescriptions compared to their ideal prescribing rate.

Infection	Percentage of patients prescribed antibiotics (%)	
	Actual	Ideal
Uncomplicated acute cough	41	10
Bronchitis	82	13
Sore throat	59	13
Rhinosinusitis	88	11
Acute otitis media (children/young adults)	92	17

Patient compliance is another factor to consider when discussing misuse of antibiotics. Patients may discontinue treatment when they feel that their health has returned, though one of the biggest reasons for non-compliance is patient fear of side-effects caused by extended use of the drugs (Tong *et al.* 2018). Withdrawing from treatment, before the course of antibiotics has been completed, exposes organisms to sub-lethal concentrations of drugs and leads to acquired resistance (Niederman 2005).

Another recognised factor to consider is the lack of regulatory and legislative controls in many countries, which would normally govern the distribution of antimicrobials (Michael *et al.* 2014). In developing countries, where healthcare is not provided universally, antibiotics are often uncontrolled, with regulatory guidelines varying from country to country (Zaman *et al.* 2017). Self-medicating with antibiotics is a regular occurrence in developing countries where prescriptions are not necessary and supply chains are not controlled (Ayukekbong *et al.* 2017)

1.1.2.2 Extensive agricultural use

The majority of antimicrobials used in agriculture are similar, if not identical, to those used clinically in the human population, and the food-chain is reported to be the primary route of transmission of AMR organisms (Zaman *et al.* 2017). Antibiotics used at sub-lethal doses are used in agriculture, farming, fisheries and crops to treat infections, prevent disease and promote growth. In animals this leads to gut microflora developing high resistance and becoming a reservoir of AMR organisms (Gupta and Deka 2018). Although the use of growth promoter antibiotics has been banned in Europe since 2006 (Prestinaci *et al.* 2015), the US are only more recently following suit. In contrast to this, places like China, India, Pakistan and Egypt, are showing an inflation in antibiotic use in animals (Anomaly 2019).

1.1.2.3 Availability of few new antimicrobials

Bacteria have historically become resistant to bacteria within ~5 years of a new antibiotic being introduced and/or used (Figure 1.3). This quick turnover

of new antibiotics has been detrimental to our repertoire of effective drugs. This, in combination with the lack of novel antimicrobials reaching end stage, often due to a lack of funding or incentive, is another driver of resistance. The sheer nature of antibiotic consumption, which is usually a short-course treatment, is not usually motivation for drug companies to develop these drugs which will not bring them much revenue in the long term (Gould and Bal 2013). Furthermore, many of the drugs used are mass produced at low prices, therefore many drug companies are apprehensive to invest in new drugs as they fear they will incur million dollar losses (Ventola 2015). The Food and Drug Administration (FDA) have regulatory strategies in place, aimed at fast-tracking the development of novel antimicrobials in their later stages of research, though these often overlook the smaller companies who lack the funds to reach the later stages of research required to benefit (Simpkin *et al.* 2017).

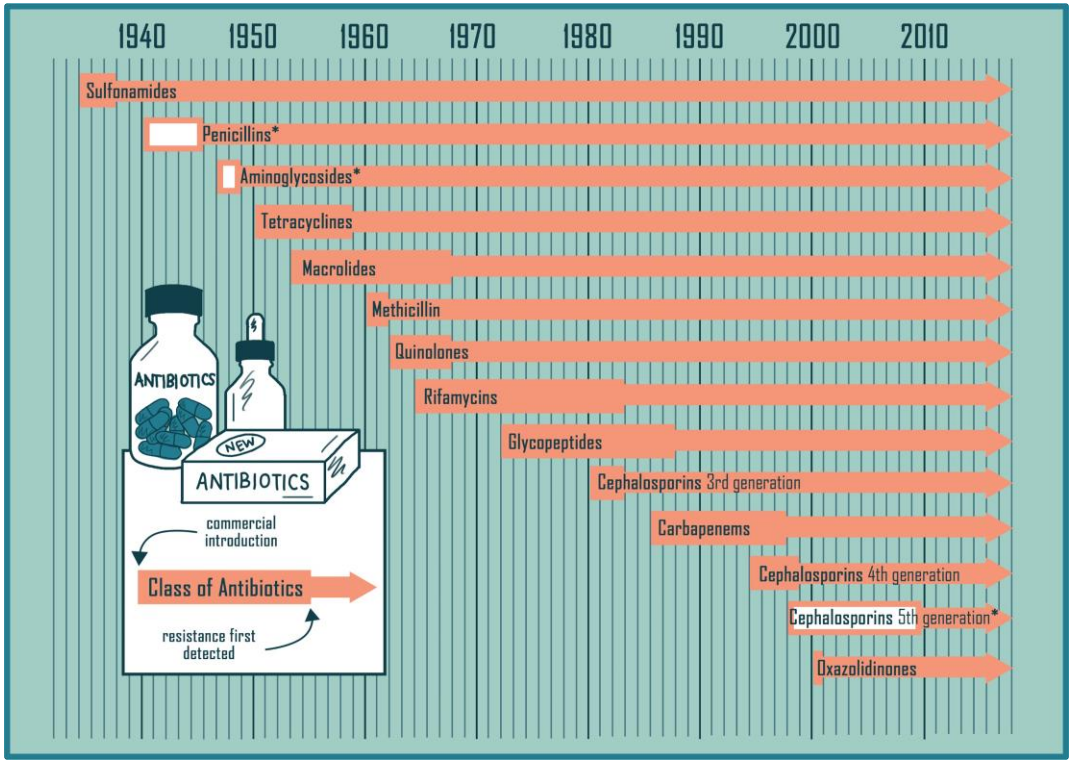


Figure 1.3 – First Indication of Bacterial Resistance Against Antibiotics

Timeline showing the dates of first introduction of a class of antibiotic and first detection of resistance. *Clear boxes indicate resistance before introduction; bacteria exhibited resistance to penicillin, aminoglycosides and 5th generation cephalosporins before their commercial release. (Image reproduced from Harbarth *et al.* 2015; CC BY 4.0)

1.1.3 Bacterial mechanisms of resistance

Resistance relies on two factors: the antimicrobial which is effective against susceptible organisms and selects resistant ones; and the resistance mechanisms present in the organism selected by the drug (Levy and Bonnie 2004). Resistance emerges when these two factors come together. There are many described mechanisms of resistance, and unfortunately no antibiotic has avoided one (Bonomo and Rossolini 2008). The basis of AMR can be simplified to fall into two categories: genetic or mechanistic (Munita and Arias 2016), but often these two categories overlap and/or influence each other. Genetic resistance can be intrinsic or acquired. Intrinsic mechanisms are already present in the genetic composition of bacteria, whereas acquired resistance is usually obtained by horizontal gene transfer via plasmids, bacteriophage, naked DNA, transposons or integrons (Levy and Bonnie 2004, Peterson and Kaur 2018). Intrinsic mechanisms include generic efflux pumps capable of pumping antimicrobials out of the cell, inactivation enzymes capable of incapacitating the drug, and permeability barriers that will prevent the penetration of drugs (Fajardo *et al.* 2008, Blair *et al.* 2015). Acquired mechanisms include plasmid encoded efflux and enzymes for modification of drugs (Peterson and Kaur 2018). Bacteria can also use alternative metabolic pathways to those targeted by the drug (Tenover 2006), can prevent binding of drugs via target modification (Miller 2016), or over-express the target enzyme to negate the inhibitory effects of the antibiotic (Palmer and Kishony 2014). Another bacterial mechanism of resistance that should be considered is the ability to form a biofilm, and its resistance can be attributed to physical, physiological and genetic mechanisms (Ciofu and Tolker-Nielsen 2019).

1.1.4 Biofilms

It was once believed that bacteria only acted singularly in a planktonic state, but it is now understood that this is not the case. Biofilms are dense populations of bacteria, which are irreversibly attached to a surface, and protected by a robust exopolymeric matrix. Biofilm formation is often thought to be the primary underlying cause for the failure of an antimicrobial agent, with 65-80% of all infections believed to be biofilm-related (Coenye and Nelis 2010). Cells existing as a biofilm often have an increased resistance to microbial agents of up to 10–1000 times (Mah and O'Toole 2001).

Bacteria instigate biofilm formation and communicate via quorum sensing (Gerdt and Blackwell 2014). It is described as an intercellular chemical signalling mechanism that is used by bacteria to monitor cell population and density. Biofilms are cell density dependent and require sufficient quantities to induce QS signal accumulation and thus QS gene expression. Many of these genes activated by QS are group beneficial and aid in the secretion of proteases, siderophores and toxins (Gerdt and Blackwell 2014).

1.1.5 Biofilm formation

The formation and survival of biofilms can be broken down into several steps, usually attachment, growth, maturation and detachment (Figure 1.4; O'Toole 2003). Initial attachment relies on many factors for success, growth and maturation. Biofilms need a continued supply of nutrients, flow of waste and efficient communication within the biofilm, and effective survival requires the detachment of cells to begin the cycle again. Furthermore, naturally occurring biofilms will often consist of a mixed-species arrangement (Kommerein *et al.*

2018). This interspecies arrangement involves communication (QS), metabolic cooperation, and interactions can be competitive or synergistic (Elias and Banin 2012).

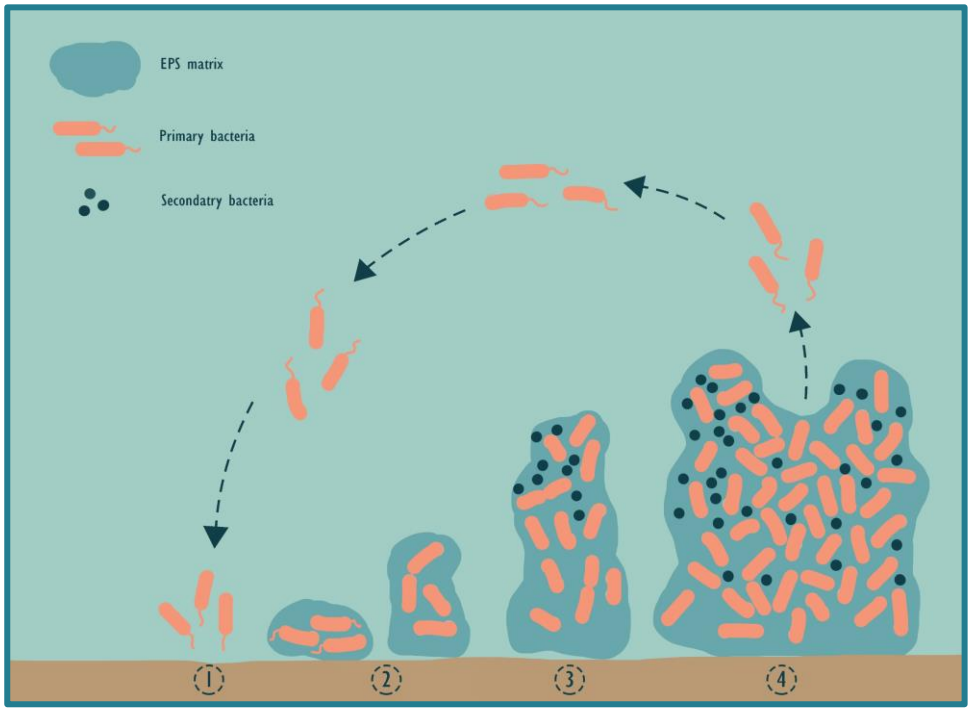


Figure 1.4 – Stages of Biofilm Growth.

Diagram showing an overview of biofilm formation. Formation begins with reversible attachment (1), followed by irreversible attachment, cell division and EPS excretion (2). A secondary bacterial species may be introduced as the biofilm matures (3), before critical mass is reached, and cells are released (4). Image created by author.

1.1.5.1 Attachment

Initial attachment relies on a surface, which would ideally be hydrophobic with nano or micro-scale roughness, and triggers physiochemical bacterial surface detection factors which administrate adherence (Cortés *et al.* 2011). Almost all natural surfaces have an overall negative charge, which primarily acts

repulsively towards the electrostatic charges in bacterial adhesion, thus adherence must overcome this using attractive Lifshitz-van der Waals, Lewis acid-base, hydrophobic, and other specific interaction forces (Van Merode *et al.* 2006). Bacteria can be transported to a surface via Brownian motion, specific gravity sedimentation or convective transport in a bulk fluid (Palmer *et al.* 2007). Initial formation begins with polar attachment of planktonic bacteria which at this stage is reversible, followed by movement of cells to align themselves flat to the surface and resist attempts of removal (Armbruster and Parsek 2018). In cases where bacteria possess flagella for motility, repression of the flagellum is often initiated after attachment, mediated by the cytoplasmic signalling molecule cyclic diguanylate, and supporting the formation of a non-motile aggregation of cells (Guttenplan and Kearns 2013). Irreversible cell attachment involves robust cell to cell organisation with binding proteins, hydrolysation of cell adhesion molecules by enzymes and protein adsorption (Pavithra and Doble 2008). Furthermore, all of the above mentioned processes are influenced by flow, carbon and oxygen levels, pH, nutrient availability and temperature (O'Toole *et al.* 2000, Toyofuku *et al.* 2016). Once bacteria have overcome this process of biofilm formation, growth and maturation can begin.

1.1.5.2 Growth and maturation

Increased quorum sensing, development of micro-colonies and the formation of extracellular polymeric covering mark the beginnings of biofilm growth, which eventually leads to a 3-dimensional structure of cell clusters (Toyofuku *et al.* 2016, Arunasri and Mohan 2019). Cells in micro-colonies begin to actively replicate by cell division (Toyofuku *et al.* 2016), generating

extracellular components which form a glycoprotein and glycolipid covering, following interaction with organic and inorganic materials surrounding them (Dunne 2002). The polymer matrix, or exopolysaccharide matrix (EPS), can be described as a gel like substance with high water content, which protects microbial cells from desiccation, among other purposes (Carpentier 1993). EPS are biopolymers consisting of polysaccharides, proteins, glycoproteins, glycolipids and extracellular DNA (Flemming *et al.* 2007); it has channels that facilitate the transportation of water, nutrients and removal of waste (Arunasri and Mohan 2019); and its quantity varies throughout maturation, often doubling in mass when reaching maturation (Jiao *et al.* 2010), but is also dependent on environmental factors and the strain of bacteria forming the biofilm (Harmsen *et al.* 2010). The structure of a mature biofilm largely depends on location, constituent organisms and nutrient concentration, and its structure can represent a homogeneous layer, dispersed micro-colonies, or protruding cell clusters (Reisner *et al.* 2003).

When a high density of cells is reached, chemical signals which are recognised by receptors within the same cells, known as autoinducers, are released. These chemical signals were originally thought to be released as siderophores, antibiotics, or as waste products (Hense and Schuster 2015). Acyl-homoserine lactones (AHLs), oligopeptide autoinducers, and autoinducer 2 (AI-2; furanosyl borate diester) are all recognised autoinducer molecules that have been found in bacteria (Hense and Schuster 2015). When the level of autoinducers reaches a critical level, bacteria respond with repression or expression of target genes (Wolska *et al.* 2016). Gene expression can benefit the biofilm by increasing virulence, promoting genetic transfer, upregulating

biofilm EPS production, upregulating efflux pumps, and contributing to resistance to stressors (Cortés *et al.* 2011, Butt and Khan 2015, Subhadra *et al.* 2016). Once maturation is reached, cells become inactive and die due to lack of nutrients, pH fluctuations, oxygen deprivation or poisoning by toxic accumulations of waste (Dunne 2002). At this point, biofilms will initiate a detachment and dispersal process to support survival.

1.1.5.3 Detachment and dispersal

Dispersal of a biofilm can occur after a number of processes take place. Cells could cease synthesis of biofilm matrix compounds, active degradation of the matrix could occur, or disruption of covalent bonds between matrix components could all accomplish detachment and dispersal (Solano *et al.* 2014). Bacteria may leave a biofilm by either desorption, detachment or dispersion, which are all classified as methods of “escape” (Davies 2011). These mechanisms can also be classified into active or passive, where active escape is initiated by the bacteria and passive escape is determined by external forces such as collision forces, predation, fluid shear, and human intervention (Kaplan 2010), formally known as abrasion, grazing, erosion, and sloughing, respectively (Petrova *et al.* 2016). Active escape mechanisms are usually induced by the selection pressures experienced by bacteria and the inane strategy for survival (Davies 2011). Once the biofilm has reached a critical mass, the outermost layer experiences dynamic equilibrium and planktonic cells are generated (Dunne 2002). The release of cells from the margins and outermost layers of a biofilm is the more commonly observed method of escape detachment, although cells may escape from the interior of

the biofilm which is known as dispersion (Davies 2011). The cells released can move freely, colonise surfaces and begin the cycle over again (Dunne 2002).

1.1.6 Biofilm tolerance

Biofilm tolerance to antimicrobials can be explained primarily by the failure of the antimicrobial agent to penetrate the biofilm, largely due to the presence of an EPS layer that is secreted when the biofilm is maturing (Butt and Khan 2015). This EPS acts as a preliminary barrier that will delay or completely prevent penetration by antimicrobial agents, although studies have indicated that this feature is often not alone in preventing infiltration (Mah and O'Toole 2001). Another cause is the slow growth rate. When biofilms are forming there is inevitably nutritional limitation and this stressor causes biofilms to exhibit a slower growth rate, which has been connected to an increased resistance as the bacteria approach stationary phase (Evans *et al.* 1991). Wentland *et al.* (1996) implemented a staining technique that was used to visualise the different growth rates within a bacterial biofilm and found that slow growth was apparent within the denser sections of biofilm, and bacteria exposed to the bulk medium showed faster growth rate. Williamson *et al.* (2012) concluded that cells deepest within a thick biofilm were in a viable, but antibiotic-tolerant, slow growing state. The general stress response can also be a significant indicator of increased resistance when in a biofilm. Stressors induce many physiological changes which act to protect the cell from starvation, osmolarity changes, pH shifts, heat shock or DNA damage (Hengge 2014).

1.2 Combatting AMR & current status

Directly addressing the drivers of resistance, as mentioned before, are the steps necessary to combatting AMR. Improving education and knowledge on AMR, global interventions to reduce the overuse and misuse of the antibiotics we have, and investments and support to boost the search for novel or alternative antimicrobials are just a few ways we can combat the crisis. Some countries sought to combat the threat of AMR as early as the 1990s (Harbarth *et al.* 2015), but it is not until more recently that other countries have joined suit. The UK has had anti-AMR strategies in place since 2000 (Mayor 2019). In 2014, David Cameron, Prime Minister of the United Kingdom, enlisted the help of economics expert Jim O'Neill to evaluate the AMR situation (O'Neill 2014). In 2016, Barack Obama, president of the United States, nearly doubled the US funding for combatting and preventing AMR, pledging to invest $1.2 billion (Obama White House 2015). The report put forward by Jim O'Neill indicates 10 interventions that need to be carried out without delay, in order to combat AMR. These 10 steps include: increasing global awareness of AMR using campaigns and programmes; improving hygiene and sanitation to prevent infection spread; reduce the use of antimicrobials in agriculture; improve surveillance of resistance and antimicrobial consumption; renew efforts to search for rapid diagnostic technology; use vaccines and alternatives to antibiotics; improve incentives for working in the field of infectious diseases; increased funding for non-commercial research; promote investments into new drugs and improve existing ones; and generate a global alliance in combatting AMR. In 2019, the UK published its most recent 5 year strategy to combat AMR (HM Government 2019). Data from the previous 5 year plan,

spanning 2013-2018, demonstrate a 7% decrease in human antibiotic consumption and a 40% decrease in sales of antibiotics for agriculture. Despite the reduction in antibiotic sales, blood stream infections caused by resistant organisms increased 35% over the 2013-2018 time frame (Courtenay *et al.* 2019). The work within this thesis will primarily focus on the search for alternative antimicrobials, specifically in those that are effective against biofilms. Potential options for novel antimicrobials include the use of natural compounds such as those derived from plants (essential oils, phenolics, lectins and polyacetylenes), animals (lactoferrin, chitosan and lysozymes), bacteria (bacteriocin, reuterin), algae, and fungi (Cowan 1999, Gyawali and Ibrahim 2014). Alternatives may also include prebiotics and probiotics, drugs that target communication within bacterial communities, or antibody based drugs (Harbarth *et al.* 2015). Recent approaches include exploring peptide based antibiotics (Roshan *et al.* 2018), renewing phage-therapy based techniques (Kortright *et al.* 2019), employing metal, polymeric and lipid based nanoparticles as antimicrobials (Lakshminarayanan *et al.* 2018), developing nanohybrid combinations of silica and antibiotics (Mosselhy *et al.* 2018), and the sequencing of prokaryote genomes to discover novel antimicrobial molecules (Tracanna *et al.* 2017). Bacterial biofilms can be combatted with several different aims in mind: prevent initial contamination; minimise initial attachment; or penetrate the preformed biofilms and eradicate the associated cells (Donlan 2002).

The research put forward in this thesis will also contribute to several aspects of the 10 interventions outlined by O'Neill (2016). It will address the need for novel antimicrobials, contributing to the improvement of hygiene and

sanitation, and potentially improve existing antimicrobials. It will do this by exploring essential oils as a novel antimicrobial that could contribute to combatting AMR.

1.2.1 Essential oils

Essential oils (EOs) are naturally occurring products of plants, which can be extracted from the leaves, petals, stems, seeds, or roots of plants (Butnariu and Sarac 2018). The term “essential oil” is thought to have come from the Swiss alchemist Paracelsus von Hohenheim in the 16th century (Nazzaro *et al.* 2017), who used the term “*Qunita essentia”* to describe plant products produced via distillation (Guenther 2013). However, the use of oils extracted from plants has been documented much earlier than this (Baser and Buchbauer 2015). As early as 4500 B.C., ancient Egyptians recognised the therapeutic potential of oils extracted from plants (Boire 2013), and regarded them so highly that King Tutankhamen was buried with approximately 350 litres of aromatic oils (Narayanasamy *et al.* 2019). China’s use of herbal medicine dates back to 3000 B.C., during the reign of Shen Nong Shi, whose work with medicinal plants is thought to be one of the earliest medical compendiums in China (Boire 2013). Commercial production and use of EOs is widespread globally, with over 100 countries producing EOs for decades (Govindasamy *et al.* 2013). However, of the ~3000 EOs known, only about 300 are commercially used (Ghabraie *et al.* 2016). The oils most popular in the industrial sector include: orange, mint, eucalyptus, citronella, peppermint, and lemon, whilst oils most commonly intended for domestic use include: lavender, chamomile, peppermint, tea tree oil, eucalyptus, geranium, jasmine,

rose, lemon, orange, rosemary, frankincense, and sandalwood (Barbieri and Borsotto 2018).

1.2.1.1 Extraction and composition

Three methods are known to be used to extract essential oils from plants including expression, hydro-distillation and dry distillation (Baser and Buchbauer 2015). The most common of these extraction methods, hydro-distillation, or steam distillation as it is otherwise known, is used throughout commercial production of EOs on industrial scales (Barreto and Coelho 2015). Extraction is usually expensive because of the relatively minimal return of final product from the raw material (Butnariu and Sarac 2018). For example, Semeniuc *et al.* (2017) found that parsley, basil and thyme yielded 0.16%, 0.4% and 2.2% oil per gram of raw material, respectively. Soni *et al.* (2016) found that nutmeg yielded an average of 5.93 mL per 100g dry weight, López *et al.* (2018) obtained a 1.8% yield from lemongrass, and Zheljazkov *et al.* (2013) extracted a maximum yield of 6.8% oil from lavender. Many factors have an influence on yield of oil from plants including: the species of plant; the part of the plant the oil is extracted from; the drying conditions; the location of plant growth; the level of milling carried out on dry matter; and distillation times (Wang *et al.* 2009, Zheljazkov *et al.* 2013, Asbahani *et al.* 2015, Baser and Buchbauer 2015, Bowes and Zheljazkov 2019). Chemical composition of the oil is also dependent on many of these same factors (Eslahi *et al.* 2017), as well as seasonal variations (Zouari-Bouassida *et al.* 2018), maturity of the plant, and genetics, although the factors effecting composition and yield are often interconnected and influence each other (Dhifi *et al.* 2016). An essential

oil is a complex mixture of highly volatile substances (Butnariu and Sarac 2018). The complexity can often reach over 300 different compounds, which belong to several different chemical classes including: alcohols, ethers, aldehydes, ketones, esters, amines, amides, phenols, heterocycles, and terpenes (Dhifi *et al.* 2016). Terpenes are the most represented family of compound within EOs and can be divided into monoterpenes, sequiterpenes and diterpenes, and also categorised as cyclic or acyclic (Buckle 2015). Because of the highly complex composition of EOs it is very difficult to replicate them synthetically in the lab (Butnariu and Sarac 2018).

1.2.1.2 Known uses

In nature, EOs have many roles in plant life. They are thought to protect the plant from disease, deter insects which may act as carriers of infection, repel predators or even entice pollinators (Nazzaro *et al.* 2017). Many people associate essential oils with aromatherapy, an alternative or complementary treatment that uses EOs (Lee *et al.* 2012). Populations from Egypt, China and India have been using EOs in aromatherapy for over 6000 years (Ali *et al.* 2015) and it has been described in cancer care (Reis and Jones 2017), reduction of anxiety (Muzzarelli *et al.* 2006) and sleep improvement (Lin *et al.* 2019). EOs, alone or in combination, have been used extensively for the treatment of wounds, inflammation, cystitis, rheumatic joints, skin sores, bleeding, fungal infections, burns, pharyngitis, syphilis, and leprosy (Narayanasamy *et al.* 2019). Commercially, EOs are usually destined for food and drink companies, perfume and fragrance companies, cosmetics, personal

care products and pharmaceuticals (Govindasamy *et al.* 2013). However, EOs have recently gained a lot of attention due to their antimicrobial properties.

1.2.1.3 Essential oils as antimicrobials

EOs have been used medicinally for many years and are widely reported as possessing antibacterial, antifungal and anti-plasmodial properties (Utchariyakiat *et al.* 2016). EOs have been described as one of the most promising natural products to combat fungal infections (Nazzaro *et al.* 2017). Work by Zabka *et al.* (2014) found that the antifungal activity of some essentials were similar to that of synthetic fungicides, and after an extensive review of the antifungal properties of essential oils reported in the literature, Whiley *et al.* (2018) found that clove oil was most researched and was a potent antifungal agent, as well as thyme, tea tree, oregano and citrus oils.

The antiviral efficacy of EOs has also been evaluated. Brochot *et al.* (2017) found that blends of EOs were active against influenza virus and herpes simplex virus 1 (HSV-1), whilst Astani *et al.* (2011) revealed that EO from star anise was highly effective against HSV-1.

EOs are extensively reported for their activity against both Gram-negative and Gram-positive bacteria in both motile and sessile states (Millezi *et al.* 2016). Janssen *et al.* (1986) screened 53 EOs for antibacterial activity against pathogens such as *E. coli, P. aeruginosa, C. albicans, B. subtilis* and *S. aureus,* and found that all of the oils showed activity against at least 1 of the organisms tested. In other studies, thyme, oregano, tea tree, cinnamon, lemon grass, bay laurel, lemon myrtle, clove, and rosewood EOs showed antimicrobial effects against *E. coli* at concentrations of <1%, with some oils

effective at concentrations as low as 0.02% (Nazzaro *et al.* 2019). Fine *et al.* (2007) found that *in vivo* tests evaluating the use of an EO-containing mouth rinse revealed significant reduction of oral pathogenic bacteria such as *Porphyromonas gingivalis, Fusobacterium nucleatum* and *Veillonella* sp*.,* after 2 weeks of use. EOs have also been shown to have synergistic effects when used in combination with antibiotics and even prevent transmission of resistance (Mulani *et al.* 2019). EOs have also been well documented for their antibiofilm activities (Budzyńska *et al.* 2011, Saviuc *et al.* 2015, Artini *et al.* 2018, Kuhn *et al.* 2019, Mittal *et al.* 2019).

EOs have even been studied *in vitro* for their potential use in treating cancer, for example, Oliveira *et al.* (2015) found that marigold EO showed no cytotoxicity in tumour cell lines. Because of the known volatility of EOs, their vapour phase has also been investigated for antimicrobial properties. In 1960, Maruzzella and Sicurella (1960) studied 133 EO vapours for antibacterial activity and found that thyme, cassia, cinnamon, origanum, and cherry laurel EO vapour inhibited a broad spectrum of bacteria. More recently, EO vapours have been used to inhibit moulds in food products (Ji *et al.* 2019); eradicate pneumonia causing bacteria (Houdkova *et al.* 2018); and combat biofilm forming bacteria (Benzaid *et al.* 2019).

1.2.1.4 Modes of action of essential oil

The antimicrobial activity of EOs against organisms can be related directly, but not exclusively, to their composition, configuration, volume and interactions with the pathogen. EOs can affect one or multiple targets within microorganisms (Dhifi *et al.* 2016) and their mode of action as whole oil is most

likely to be attributed to their composition. Essential oils are usually described as having 2 or 3 major constituents, often representing up to 85% of the entire composition, and usually responsible for the biological activity the oil exerts (Chouhan *et al.* 2017). However, it has been shown that the minor components often have a role to play as well (Feyaerts *et al.* 2018). The chemical class of the antimicrobial constituent typically eludes to the mechanism of action of the oil against bacteria (Swamy *et al.* 2016). Oils with a high proportion of aldehydes and phenols usually have a strong antimicrobial activity, whilst those with ketones, esters or terpene hydrocarbons have a weaker activity, or lack any antimicrobial efficacy at all (Bassolé and Juliani 2012). One of the most important characteristics of oil EOs is their hydrophobicity, which results in increased cell permeability and leads to cell leakage (Dhifi *et al.* 2016). The majority of EOs act on the cell wall or membrane of bacteria and can cause the release of lipopolysaccharides from the membrane (Faleiro 2011). Intracellular and external ATP balances are likely to be affected as well as protein synthesis, pH fluctuation, intracytoplasmic changes such as coagulation of cytoplasmic material, DNA disruption and inhibition of quorum sensing (Faleiro 2011, Lopez-Romero *et al.* 2015). Processes influencing energy conversion, nutritional balances, structural macromolecule synthesis and growth regulation have all been reportedly effected by EOs (Swamy *et al.* 2016).

Phenolic compounds, for example carvacrol, eugenol and thymol, have reportedly affected the cytoplasmic membrane, proton forces, electron flow, active transportation and cell content coagulation (Dhifi *et al.* 2016). It is also reported that components contributing to the composition of EOs may have a

synergistic, additive or antagonistic effect on one another (Pei *et al.* 2009), therefore it is essential to have a clear understanding of the composition of any particular EO, to have a clear understanding of mode of action.

1.2.1.5 Safety

The majority of EOs are classified by the Food and Drug Administration as generally recognised as safe (GRAS), and several have been approved for use in food applications and for consumption (Ali *et al.* 2015). The most common adverse reaction encountered from EOs is typically a result of individual hypersensitivity, and risks are usually controllable (Rather *et al.* 2016). Common complaints caused by EO interactions are related to eye, mucous membrane and skin irritation or sensitization (Ali *et al.* 2015). However, more serious cases of toxicity have been reported where vomiting, epigastric pain, diarrhoea, convulsions, renal failure, and central nervous system depression have been observed (Eisenhut 2007). The toxicity of an oil can be increased by improper storage, which could lead to photoisomerisation, photocyclisation, oxidation, peroxidation and decomposition of alcohols, ketone hydrolysis, and overall degradation, potentially reducing the safety of an EO (Sarkic and Stappen 2018). Whilst toxicity testing on EOs is often limited to specific named EOs, several studies have investigated the toxicity of oils *in vivo* in rodent and small mammal models. For example, Mekonnen *et al.* (2019) found that lavender EO was not toxic when evaluated orally and dermally in mice and rabbits. *In vitro* studies have also been used to access toxicity of oils in human cell lines. Puškárová *et al.* (2017) looked at the toxicity of several essential oils when introduced to human embryo lung cells and

found that none of the oils induced any genotoxic effects. There have been reports of accidental or intentional ingestion of EOs in humans, although in cases where citronella EO was ingested no toxicity was shown (Vigan 2010), and where near fatal incidents have occurred, consumption was thought to be at extremely high doses (Nath *et al.* 2012). With safety in mind, it is sensible to suggest that toxicity of most oils can be circumvented by avoiding ingestion, diluting oil for topical applications and ensuring proper storage (Hammer *et al.* 2006).

1.3 Summary and aims

The increasing development of resistant bacteria is putting an enormous strain on the current repertoire of antimicrobials. This global crisis of AMR is the result of years of antibiotic abuse and misuse, among other drivers of resistance. AMR is a financial burden to the whole world and is already having a catastrophic effect on the population. Concerted efforts must be made to develop new policies of use, continue research efforts, and develop novel interventions to mitigate this threat. Combatting those bacteria most highly implicated in this crisis, and specifically focussing on the mechanisms of resistance that they possess, is fundamental in the development of successful antimicrobials. EOs are a naturally occurring product of plants and have historically been used as antimicrobials, but many EOs remain neglected in research.

The aims of this thesis are to explore EOs as alternative antimicrobials, to investigate their anti-biofilm activity and to evaluate their potential to contribute to the control of AMR. These aims hope to support or refute the hypotheses

that EOs will be effective antimicrobials with the ability to combat biofilms, EOs could be successfully incorporated into future development of new antimicrobials that can aid in the reduction of AMR, and that investigation into EOs as novel antimicrobials is warranted.

CHAPTER 2

2 MATERIALS AND METHODS

2.1 Chemicals and media

All chemicals and media used in this study were purchased from Sigma Aldrich (Dorset, UK), Oxoid (Hampshire, UK) or Fisher Scientific (Leicestershire, UK), unless otherwise specified. All media were made using deionised water and sterilised by autoclave, unless otherwise specified.

2.2 Maintenance and growth of bacteria

Bacteria tested throughout this study were obtained from the microbiology culture collection at the University of the West of England, Bristol, UK. The strains used were: *Escherichia coli* (Strain B; ATCC 23848), *Escherichia coli* (NCTC 9001), *Escherichia coli* (ATCC 10536), *Staphylococcus aureus* (ATCC 6538), *Staphylococcus aureus* (NCTC 12981), *Pseudomonas aeruginosa* (PAO1), *Pseudomonas aeruginosa* (NCTC 8505), *Pseudomonas aeruginosa* (ATCC 15442), Hospital Acquired Methicillin-Resistant *Staphylococcus aureus* (MRSA) isolate, Methicillin-Susceptible *Staphylococcus aureus* (MSSA; NCTC 13297), *Acinetobacter baumannii* (NCTC 12156), *Acinetobacter baumannii* (ATCC 17978) and *Enterococcus hirae* (ATCC 10541). All bacteria were stored on beads (Microbank, Pro Lab Diagnostics, Canada) at -80°C and revived on nutrient agar (NA; Oxoid, Hampshire, UK) slopes at 37°C for 24 h. These slopes were kept at 4°C as working cultures for a maximum of 6 weeks. Overnight broth cultures were prepared using one to three colonies of bacteria from these slopes, which were added to 10 mL of tryptone soy broth (TSB; Oxoid, Hampshire, UK) and incubated at 37°C for 24 h. When required, cultures were standardised by diluting with TSB and

measuring optical density at 600 nm to obtain a reading of 0.08-0.1 (McFarland 0.5), giving a standardised inoculum of ~1-2 x 10^8 CFU mL^{-1}.

2.3 Essential oils

Fifteen essential oils were used, and included EOs from: bergamot (*Citrus bergamia;* peel), cinnamon (*Cinnamomum zeylanicum;* bark and leaf), clove (*Eugenia caryophyllus;* bud), grapefruit (*Citrus paradise;* peel), lavender (*Lavandula angustifolia;* flower), lemon (*Citrus limonum;* peel), lemongrass (*Cymbopogon citratus,* dried grass), lime (*Citrus aurantifolia,* fruit), manuka (*Leptospermum scoparium*; leaves), sweet orange (*Citrus sinensis*; peel*)*, rose geranium (*Pelargonium graveolens*; whole plant), rosemary (*Rosmarinus officinalis*; leaves), North Indian rosewood (*Dalbergia sissoo*; wood), tea tree (*Melaleuca alternifolia*; leaves) and thyme (*Thymus vulgaris*; whole plant). All of these oils were donated by Amphora Aromatics Ltd, Bristol, UK for research purposes, with the exception of cinnamon leaf oil which was purchased separately and manufactured by Natural by Nature Ltd, UK. Oils were considered to be at 100% concentration in their neat form and subsequent dilutions stated as a percentage (v/v) reflects this.

2.3.1 Oil analysis

Gas Chromatography Mass Spectrometry (GC-MS) analysis of cinnamon bark EO was performed using methods adapted from Adukwu *et al.* (2012). Cinnamon leaf EO was also analysed as a comparator to the bark EO to identify the differences in the components as these EOs are obtained from different parts of the same plant. GC-MS analysis used an Agilent 6890N

Network Gas Chromatograph system and 5973 Network Mass Selective Detector (Agilent Technologies, USA). Samples were dissolved in acetone with a 1 µL injection volume. Inlet temperature was 300°C with a split ratio of 10:1. Temperature of the transfer line was 300°C and solvent delay was 3 min. The carrier gas was helium with a column flow rate of 1 mL min^{-1} and operating at constant flow. The oven temperature started at 50°C and was held for 2 min, then increased until 280°C was reached at a rate of 10°C min^{-1}, with a total run time of 25 min. Compound separation was achieved with a HP-5MS column (30 m × 0.25 mm × 0.25 µm). An electron impact ion source at 230°C, and a quadrupole mass analyser at 150°C was used. Electron ionization mass spectrometric data were collected between 30 to 550 m/z. Oil components were identified by comparison of mass spectral patterns with those from the spectra from the National Institute of Standards and Technology (NIST) library (MS Search 2.0) provided by the software of the GC-MS system.

2.3.2 Preparation of essential oils (EOs)

Where EO was required at different concentrations, unless otherwise indicated, a stock solution of EO was prepared in TSB containing dimethyl sulfoxide (DMSO; Sigma Aldrich, Dorset, UK) which is a solvent used as a dispersal agent (Kačániová *et al.* 2017). Further two-fold dilutions of this stock, in TSB, produced a range of EO concentrations from 0.015% to 8% (v/v), with DMSO concentrations of 0.0098% to 5% (v/v), respectively. This produced an EO to DMSO ratio of 1:0.625, which was maintained throughout experiments in this thesis.

2.4 Biofilm growth *in vitro*

2.4.1 Microtitre plate method (crystal violet biofilm assay)

Methods for assessing in vitro biofilm growth were adapted from Stepanović et al. (2000). An overnight culture was standardised as described in section 2.2, with the exception of being diluted in TSB supplemented with 1% (v/v) glucose. Two hundred microliters of this suspension were added to each well of the microtitre plate. Three wells each were used per bacterial strain, ensuring adequate spacing between each to avoid risk of contamination. Negative control wells contained TSB with 1% glucose only. Plates were incubated at 37°C for 24 hours. The wells were then carefully aspirated and washed three times using 250µl of sterile PBS. Bacteria were fixed using 200µl of methanol for 15 minutes, wells were emptied and then air dried. After air drying, wells were stained with 250µl of 0.1% crystal violet for 5 minutes. Excess stain was removed by rinsing gently with tap water. After leaving to air dry, the bound stain was solubilised with 250µl of 33% (v/v) acetic acid. The optical density (OD) of wells in the plate was then measured using a TECAN Infinite® 200 PRO plate reader at OD_{595nm}. Biofilm growth was categorised by strength of adherence which was defined as: non-adherent, weak, moderate and strong. This classification depended on the OD values of wells and its comparison to the OD cut-off (ODc) value. ODc was defined as three standard deviations above the mean OD of the negative control. Experiments were carried out independently in triplicate and categorisation of biofilms was based on the following formulae:

$$OD \leq ODc = \ non\text{-}adherent$$

$$ODc \ < OD \leq 2 \times ODc = weakly\ adherent$$

$$2 \times ODc \ < OD \leq 4 \times ODc = \ moderately\ adherent$$

$$OD > 4 \times ODc = strongly\ adherent$$

2.4.2 CDC biofilm reactor

A Centre for Disease Control (CDC) Biofilm Reactor (CBR; model CBR 90-2; BioSurface Technologies, USA) was used to produce biofilms under high shear and continuous flow (Figure 2.1). Full specifications for this equipment and its use can be found in the manufacturer's manual. Briefly, it consists of a 1 L Pyrex beaker with an outlet port at 400 mL, resulting in an approximate working volume of 350 mL. The ultra-high molecular weight (UHMW) polyethylene ported lid holds 8 polypropylene rods, which each accommodate 3 coupons (1.27 cm width, 0.3 cm thickness). Medium is circulated through the vessel and the shear is generated by a magnetic baffle and digital stir plate. Coupons used were polycarbonate or stainless steel, and they were cleaned by sonicating for 5 min in 1:1000 Decon90 (Fisher Scientific, Leicestershire, UK), rinsing in deionised water, followed by a 5 min sonication in deionised water. Polycarbonate coupons underwent a further 2 h soak in 2 M hydrochloric acid. Coupons were left to air dry in Petri dishes lined with filter paper. The assembled CBR, including cleaned coupons, was filled with 500 mL of 300 mg L^{-1} TSB and autoclaved at 121°C for 15 min. Once cooled, the reactor was inoculated with 1 mL of standardised *P. aeruginosa* culture and placed on a digital stir plate operating at 130 rpm at room temperature. The CBR was operated in batch phase for 24 h, followed by continuous flow phase

using 100 mg L^{-1} TSB at a flow rate of 11.6 mL min^{-1} for a further 24 h. This produced standardised 48 h mature biofilms.

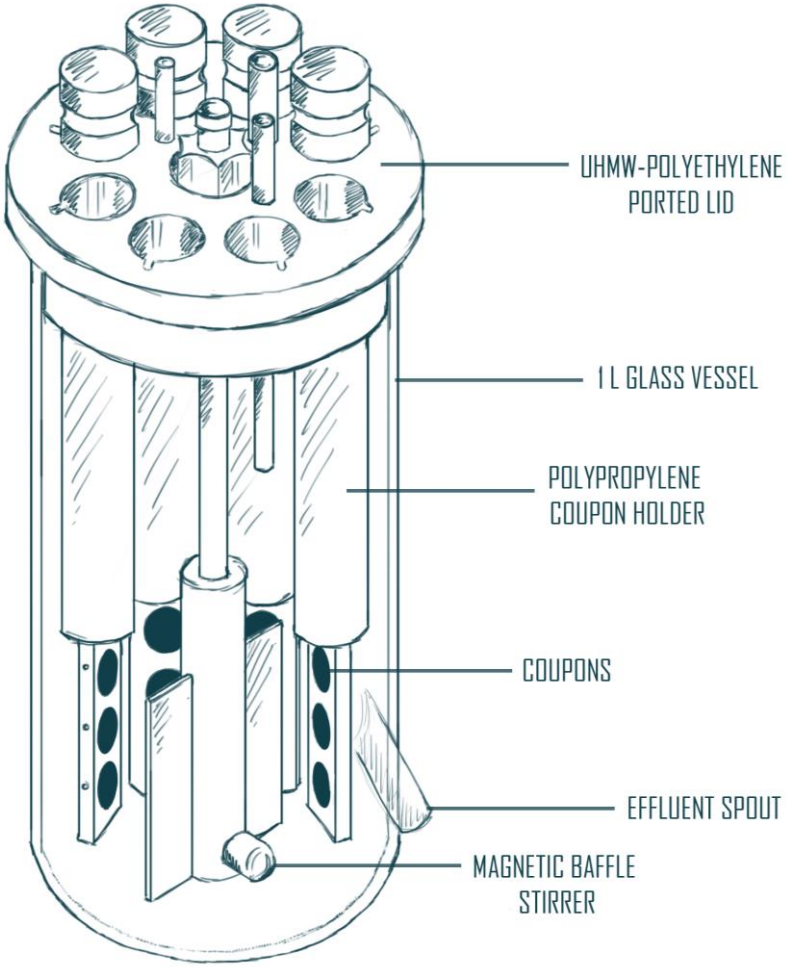


Figure 2.1 – Centre for Disease Control (CDC) biofilm reactor.
A diagram depicting the assembled vessel of the CDC biofilm reactor used to grow biofilms under continuous flow and medium-high shear force. Image created by author.

2.5 Imaging

2.5.1 Scanning electron microscopy (SEM)

Samples for SEM were prepared using the method reported by Murtey and Ramasamy (2016) and adjusted as indicated below. Briefly, small squares of poly-l-lysine coated microscope slides (Sigma Aldrich, Dorset, UK) were placed in wells of a 12-well plate. Wells were inoculated with 1 mL of bacterial samples in broth culture and left to adhere for 1 h. The squares of microscope slide were removed and placed in a new 12-well plate. The attached cells were then fixed by immersion in 4% (v/v) glutaraldehyde (Sigma Aldrich, Dorset, UK) in 0.1 M phosphate buffer (10.22 g L^{-1} Na_2HPO_4, 3.36 g L^{-1} NaH_2PO_4) for 30 min, washed with the same buffer three times and then dehydrated through a series of ethanol concentrations for 10 min each, followed by hexamethyldisilizane (HMDS; Sigma Aldrich, Dorset, UK) for 10 min two times. The squares were mounted on aluminium stubs (Agar Scientific Ltd, Essex, UK) and coated with gold in a sputter coater and viewed using a scanning electron microscope (FEI Quanta 650 FEG, Sigma Aldrich, UK).

2.6 Data analysis

The following data analysis was carried out, unless otherwise specified. Data were analysed using Microsoft Excel 2016 (Microsoft Corporation, Redmond, USA) and GraphPad Prism version 8.00 for Windows (GraphPad Software, La Jolla California, USA). Minimum level of significance was set to $P<0.05$, and where asterisks were used to indicate significance: * = $P<0.05$; ** = $P<0.01$; *** = $P<0.001$; **** = $P<0.0001$.

CHAPTER 3

3 THE ANTIMICROBIAL ACTIVITY OF ESSENTIAL OILS

3.1 Introduction

Many essential oils (EOs) are well described to have antimicrobial activity (Millezi *et al.* 2016). Despite the widespread use of EOs for multiple purposes, only a small proportion of the known EOs are used commercially (Ghabraie *et al.* 2016). Whilst it is thought that the majority of EOs act on the cell wall and membrane of bacteria (Faleiro 2011), studies assessing mode of action of individual oils are still required. Furthermore, very few studies exploring the antimicrobial activities of EOs investigate their potential for rapid killing, i.e. under 10 min contact time. Friedman *et al.* (2004) investigated the activities of EOs and their components against *E. coli* O157:H7 with incubation times of ≥5 min, and Tangjitjaroenkun *et al.* (2012) studied the antimicrobial effects of EO from *Zanthoxylum limonella* with incubation times of ≥3 min. Mayaud *et al.* (2008) assessed the inhibitory action of various EOs against bacteria with contact times of ≥5 min. However, to my knowledge, to date no study has evaluated the antimicrobial efficacy of EOs against *P. aeruginosa* with less than 5 min contact time. As part of the search for novel antimicrobials, efficacy of new antimicrobial compounds must be quantitatively assessed using antimicrobial screening assays. These often include broth dilution methods to determine the minimum inhibitory concentration (MIC), a value representing the lowest concentration of antimicrobial required to inhibit microorganism growth. Dimethyl sulfoxide (DMSO) is a clear odourless liquid which acts as an aprotic solvent (Brayton 1986). It has a strong affinity to and is soluble in water, but can also solubilise in ethanol, acetone, diethyl ether, benzene and

chloroform (Pope and Oliver 1966). It is a useful solvent for compounds such as therapeutic and toxic agents, which are not soluble in water, and is often used in place of methanol or ethanol when these two solvents are incapable of dissolving a compound of interest (Wadhwani *et al.* 2012). It is important to consider any possible effects that these supplements have on the outcome of such experiments, and whether they would interfere with the results. Therefore, this chapter first aims to investigate the potential effects of DMSO on the chosen panel of bacteria, whether it enhances or suppresses growth and if it influences the outcome of antimicrobial testing. As previously described, there is a large breadth of essential oils available to investigate, therefore this chapter further aims to assess the effects of a selection of EOs against a panel of bacteria, with the aim to streamline investigations to focus on one selected EO, exhibiting the greatest antimicrobial effects, and investigate its rapid bactericidal potential and mode of action.

3.1.1 Chapter progression

The following flow diagram depicts a summary of the progression of this chapter.

3.2 Methods

3.2.1 Test organisms and standardisation of overnight cultures

The bacteria used included *Escherichia coli* (ATCC 23848), *Escherichia coli* (NCTC 9001), *Staphylococcus aureus* (ATCC 6538), *Staphylococcus aureus* (NCTC 12981), *Pseudomonas aeruginosa* (PAO1), *Pseudomonas aeruginosa* (NCTC 8505), Hospital Acquired Methicillin-Resistant *Staphylococcus aureus* (MRSA) isolate, Methicillin-Susceptible *Staphylococcus aureus* (MSSA; NCTC 13297), *Acinetobacter baumannii* (NCTC 12156), *Acinetobacter baumannii*

(ATCC 17978). Their storage and standardisation were carried out as described in section 2.2.

3.2.2 Effect of dimethyl sulfoxide (DMSO) on bacterial growth

Methods to assess the effect of DMSO on bacterial growth were adapted from those previously described by Wadhwani *et al*. (2012). Standardised overnight cultures were further diluted in TSB (1:150) and 100 µL of this (approximately $5x10^5$ CFU mL^{-1}) was added to the wells of a 96-well microtitre plate. Concentrations of 2.5% and 5% DMSO were prepared in TSB and 100 µL of each concentration was added to the inoculated wells to give final concentrations of 1.25% and 2.5%. Microtitre plates were incubated at 37°C for 24 h. Contents of wells were diluted and 100 µL spread onto NA agar plates, which were incubated for 24 h at 37°C and then enumerated. TSB and bacterial culture in equal parts was used as a negative control. DMSO and TSB alone were also included to ensure no contamination was present. Each organism was grown in triplicate and three independent experiments were carried out. Final values are expressed as mean CFU mL^{-1}.

3.2.3 Headspace analysis of *E. coli* in DMSO by selected-ion flow-tube mass spectrometer (SIFT-MS)

Methods to assess headspace of broth culture were adapted from methods outlined by Slade *et al*. (2017). Briefly, 9 mL of 1.25% DMSO in TSB was added to sterile 250 mL glass bottles with a screw cap containing a silicone septum. A standardised overnight culture of *E. coli* (ATCC 23848) was diluted 1:150 and 1 mL of this suspension was used to inoculate the glass bottles. A

glass bottle containing only TSB with culture was used as a negative control. Bottles were incubated for 4 h at 37°C. Headspace analysis of the culture was analysed using a SIFT-MS (Instrument Science Limited, UK) in 'Full Mass Scan' mode and an H_3O+ precursor ion was used. A sterile needle attached to the SIFT-MS direct sampling inlet was used to pierce the silicon septum of the sample bottle and the sample was vented using another sterile needle (Fisher Scientific, Leicestershire, UK) attached to a 0.2 µm syringe filter (Sartorious Stedim Biotech, Germany) to allow for free-flowing headspace gases. Three analyses were carried out using repeat scans of 60 s, over a spectrum range of 10–200 m/z, and three independent experiments were carried out.

3.2.4 Quantification of metabolites produced by *E. coli* in presence of DMSO using SIFT-MS

Headspace analysis identified a dominant peak relating to the presence of dimethyl sulfide (DMS); thus, the following method was used to quantify this compound. Briefly, the headspace of the chamber was analysed using a SIFT-MS in 'Selected Ion' mode to quantify headspace concentration of DMS. H_3O+, $NO+$ and O_2+ were used as precursor ions. A sterile needle attached to the SIFT-MS direct sampling inlet was used to pierce the silicon septum of the vapour chamber and the sample was vented using another sterile needle attached to a 0.2 µm syringe filter to allow for free-flowing headspace gases. Three analyses were carried out using repeat scans of 90 s and three independent experiments were carried out. Final values were calculated as mean parts per billion (ppb).

3.2.4.1 Influence of DMSO on antimicrobial testing

The broth dilution method of antimicrobial testing was used in future chapters of this thesis to determine minimum inhibitory concentration (MIC) and used DMSO as a solvent. An alternative method was employed to evaluate whether the presence of DMSO influenced the results. The agar dilution method, which was not chosen for studies within this thesis, but widely used in other studies, does not employ DMSO as a solvent. Thus, this method was used to determine the MIC of cinnamon bark, clove and tea tree essential oil against *E. coli* B (ATCC 23848) and the results compared to those from MIC testing described in chapter 4. Methods used here were adapted from Clinical and Laboratory Standard Institute (CLSI; 2012) and Griffin *et al.* (2000). Briefly, MHA agar was prepared and supplemented with 0.5% Tween 20 (v/v). Essential oil was added to 15 mL aliquots of this molten agar, to achieve doubling concentrations ranging from 0.03% to 2% (v/v). Molten agar was mixed thoroughly, whilst avoiding production of bubbles, and poured into petri dishes and allowed to set at room temperature. A standardised overnight culture of *E. coli* B (ATCC 23848) was prepared as described in section 2.2 and further diluted 1:150 in TSB. Once the prepared agar plates were dry, plates were inoculated with 10 µL spots of diluted culture. One agar plate inoculated with the test organism in the absence of essential oil was used as a negative control with each experiment. Each plate was inoculated with three spots and three independent replicates were carried out.

3.2.5 Essential oils

The EOs used in this chapter are described in section 2.3.

3.2.6 Disc diffusion assay

Screening of EOs for anti-bacterial activity was performed using a paper disc diffusion approach adapted from the Clinical and Laboratory Standards Institute (CLSI) Antimicrobial Susceptibility Testing (AST) Standards (Clinical and Laboratory Standards Institute 2015). MHA plates were inoculated with 100 µL of standardised culture for each test bacterium, spread evenly over the entire surface of the agar using a sterile cotton swab by swabbing in three directions (Howe and Andrews 2012). The inoculum applied to each plate was ~1-2 x 10^7 CFU mL^{-1}. A volume of 10 µL of EO was used to saturate a set of six mm diameter filter paper discs (Whatman, Sigma Aldrich, UK), one of which was then placed onto the centre of each inoculated plate. Sterile discs (6mm) without EO were used as a negative control and discs containing 30 µg gentamicin (Oxoid, Hampshire, UK) were used as a positive reference. Plates were incubated at 37°C for 24 h and antibacterial activity was assessed by measuring the diameter of the zones of inhibition in mm using Vernier callipers. Three independent experiments were performed in triplicate.

3.2.7 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC of EOs was determined by the broth microdilution method as outlined by the CLSI (2012) and adapted from previously described methods (Cao *et al.* 2009). EO dilutions were prepared as described in section 2.3.2. One

hundred microliters of each EO concentration were added to wells of a 96-well microtitre plate. A standardised overnight culture (as described in section 2.2) was diluted 1:150 with TSB, and 100 µL of this was added to each well of the microtitre plate. Final concentrations of EO ranged from 0.007% to 4% (v/v) with DMSO concentrations of 0.0049% to 2.5% (v/v) respectively. Final cell density of the inoculum was approximately 5 x 10^5 CFU mL^{-1}. Microplate wells of EO dilutions without bacteria, and TSB and DMSO alone, were used as negative controls, and plates were incubated at 37°C for 24 h. MIC values were determined by the well with the lowest concentration of EO where no visible microbial growth was observed. Each concentration of EO had three replicates and experiments were independently repeated four times.

MBC was determined by spot inoculation similar to methods outlined by Adukwu *et al.* (2012). Briefly, 10 µL was pipetted from the microtitre wells showing no growth onto MHA, and incubated for 24 h at 37°C. Each concentration of EO had three replicates and experiments were independently repeated four times. MBC values were determined as the lowest concentration of EO where no growth was observed.

3.2.8 Gas chromatography mass spectrometry (GC-MS) analysis

Following determination of MICs and MBCs, cinnamon bark EO demonstrated broad-spectrum activity against all tested organisms, including *P. aeruginosa* at <4% (v/v). Thus, this oil was selected for testing rapid bactericidal effects against *P. aeruginosa*.

GC-MS analysis of cinnamon bark EO was performed as described in 2.3.1. Cinnamon leaf EO was also analysed as a comparator to the bark EO, to

identify the differences in the components, as these EOs are obtained from different parts of the same plant species.

3.2.9 Time-kill assay

Time kill kinetics were determined for cinnamon bark EO against *P. aeruginosa* PAO1 using methods adapted from Carson *et al.* (2002). Six 50 mL conical flasks containing 15 mL of TSB were inoculated with 50 µL of standardised *P. aeruginosa* PAO1 overnight culture to achieve approximately 5×10^5 CFU mL^{-1}. A 100 µL aliquot was removed from each flask and added to 9.9 mL of TSB, diluted serially and plated onto MHA to confirm starting inoculum density, representing the zero min time point (T0). Table 3.1 shows the volumes of EO and DMSO added to the flasks to achieve a series of concentrations for the time-kill assay. The concentration of cinnamon bark EO ranged from 0.125% (v/v; 1 x MIC) to 2% (v/v; 16 x MIC), with a negative control flask containing DMSO alone. After the addition of EO and DMSO, the flasks were vortexed and incubated without shaking at room temperature. At each time point the flasks were vortexed again and aliquots were removed at 2, 4, 6, 10 and 30 min and at 1, 2, 4, 6 and 24 h, diluted serially and plated onto MHA. All plates were incubated at 37°C for 24 h before enumeration of the colonies. The effect of an antimicrobial compound is considered bactericidal if a $\geqslant$99.9% decrease in the initial inoculum (i.e. a three-log reduction) is observed, as described by the CLSI (1999).

Table 3.1 – Preperation of Essential Oil Dilutions.

Volumes of EO and DMSO prepared before adding to flasks containing 15 mL TSB inoculated with *P. aeruginosa* to achieve range of final cinnamon bark EO concentrations

Concentration of EO required (v/v)	Volume of EO added (µL)	Volume of DMSO added (µL)
0%	0	187.5
0.125%	18.8	11.7
0.25%	37.5	23.4
0.5%	75	46.9
1%	150	93.8
2%	300	187.5

3.2.10 Metabolic activity assay

A triphenyltetrazolium chloride (TTC) metabolic activity assay was performed to investigate the effect of EO on metabolic activity during the time-kill assay, as described by Ahmed (2013). One hundred microlitre aliquots of the treated organisms from each time point were transferred to wells of a 96-well plate. A 5 µL volume of sterile 1% aqueous solution of TTC (Sigma Aldrich, Dorset, UK) was added to each well and plates were wrapped in aluminium foil and incubated at 37 °C with orbital shaking at 120 rpm for 24 h. TSB containing no bacteria was used as a negative control. The presence of viable bacterial cells was indicated by the reduction of the yellow TTC to a red colour. Optical density of the wells was measured using a TECAN Infinite® 200 PRO plate

reader at 595 nm. Data were normalised to T0 and expressed as a percentage relevant to this time point. All assays were performed in triplicate on three separate occasions.

3.2.11 Scanning electron microscopy (SEM)

SEM was used to assess morphological effects on *P. aeruginosa* PAO1 after treatment with cinnamon bark EO at 2% (v/v) for 10 min. Both untreated and treated samples were prepared for SEM as described in section 2.5.1.

3.2.12 Release of nucleic acids and proteins

The release of 260 nm absorbing nucleic acids and 280nm absorbing proteins from *P. aeruginosa* PAO1 after treatment with cinnamon bark EO was measured according to methods described by Miksusanti *et al*. (2008), with some adaptations. A 50 mL conical flask containing 15 mL of cinnamon bark EO at either 2% (v/v) or 1.25% (v/v) in TSB with 1.25% (v/v) or 0.08% (v/v) of DMSO, respectively, was inoculated with 50 µL of standardised overnight *P. aeruginosa* PAO1 culture. The flask was vortexed, incubated at room temperature for 2 h and then a 1 mL aliquot was removed and filtered with 0.45 µm filter (Sartorious Stedim Biotech, Germany). The absorbance of this filtrate was measured at 260 nm and 280 nm. Negative controls were treated with DMSO (1.25% v/v) alone. The absorbance of filtrate from controls without culture added were deducted from the absorbance of the respective samples with EO added. Experiments were performed in triplicate on three separate occasions, and results are expressed as mean OD of nucleic acids (260 nm) and protein (280 nm) released.

3.2.13 Data analysis

A one-way ANOVA with Tukey's multiple comparisons test was used to compare means of growth of bacteria in absence or presence of DMSO at different concentrations. A two-tailed t-test was used to identify significant differences in DMS production when in presence or absence of DMSO.

3.3 Results

3.3.1 Effect of dimethyl sulfoxide (DMSO) on bacterial growth

The effects of DMSO solvent on bacterial growth are presented in Figure 3.1. DMSO at either 1.25% or 2.5% (v/v) had little to no effect on the growth of any organism, with the exception of *E. coli* B (ATCC 23848). *E. coli* B (ATCC 23848) in the presence of either concentration of DMSO showed a promotion of growth, double that of the control, and produced an unpleasant odour after overnight incubation.

3.3.2 Headspace analysis of *E. coli* in DMSO by selected-ion flow-tube mass spectrometry (SIFT-MS)

Due to the growth promotion and odour observed for *E. coli* B (ATCC 23848), further investigations were made to analyse the headspace of the culture in the presence of DMSO. A noticeable peak at 63 m/z, coupled with identification using the built-in compound library, indicated a production of DMS that was more than 100-fold greater in the culture with DMSO present.

3.3.3 Quantification of metabolites produced by *E. coli* in presence of DMSO using SIFT-MS

Results from further quantification investigations are presented in Figure 3.2. When *E. coli* is in the presence of 1.25% (v/v) DMSO, DMS production is significantly greater than when *E. coli* is not exposed to DMSO ($P<0.05$). DMS produced in the presence of DMSO was determined to be 10434.8 ppb whilst DMS produced in the absence of DMSO was 2.3 ppb.

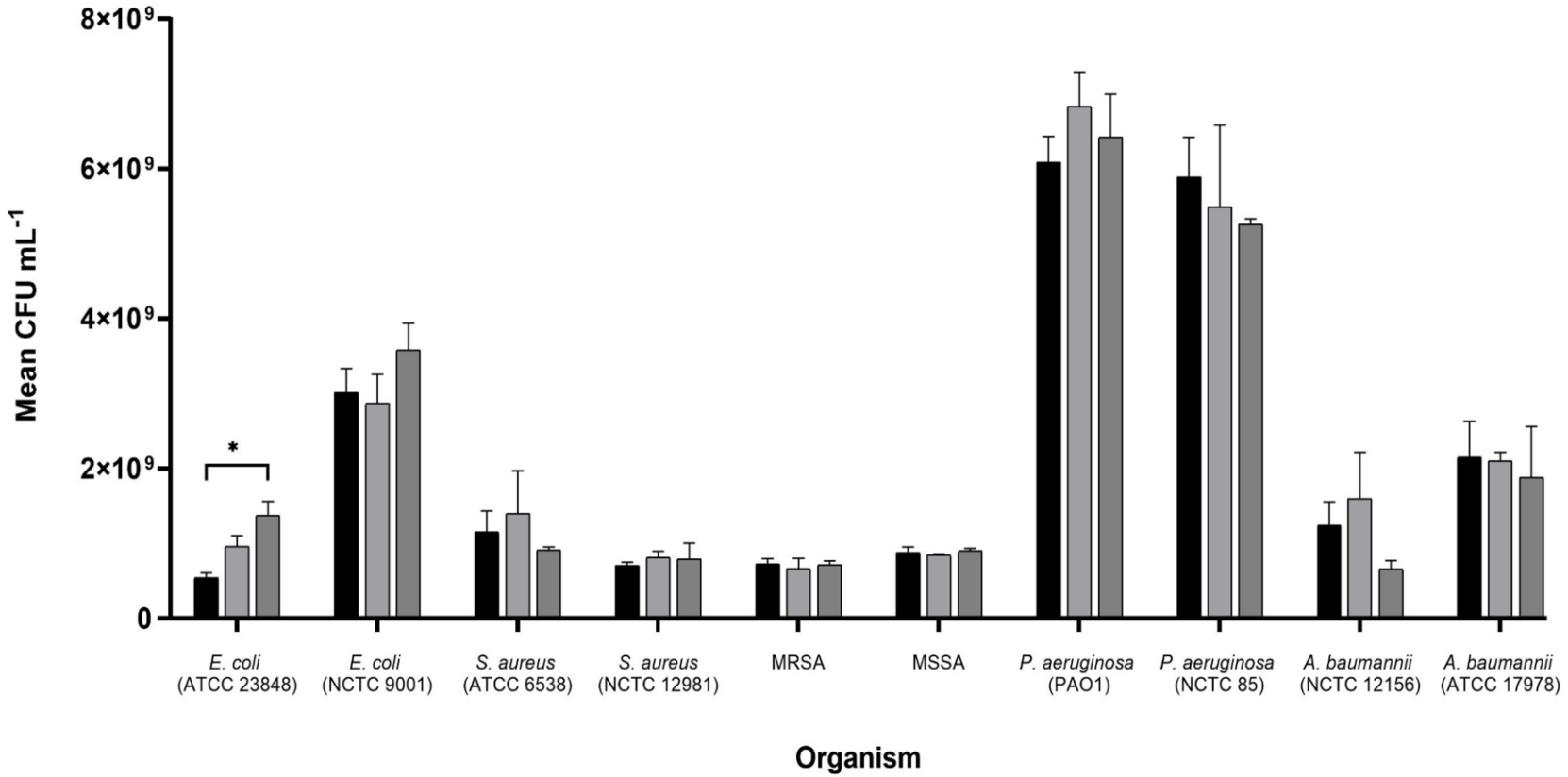


Figure 3.1 – Effect of DMSO on bacterial growth.

Mean CFU mL^{-1} of bacteria in presence or absence of dimethyl sulfoxide (DMSO). ■ control; ■ 1.25% (v/v) DMSO; ■ 2.5% (v/v) DMSO. N=3; Control = organism not exposed to DMSO; bars showing standard error; asterisk indicates significance.

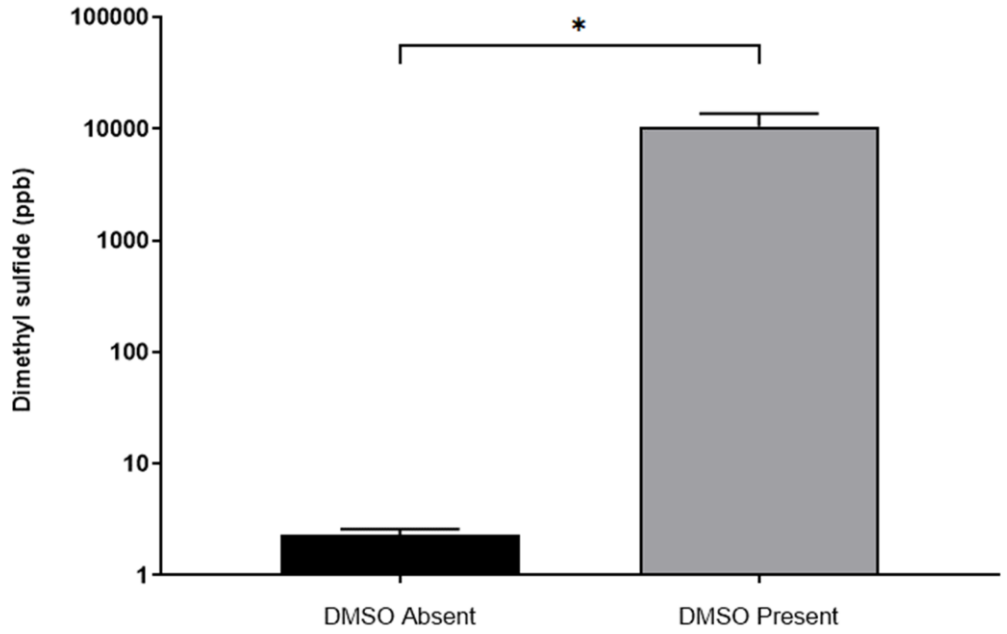


Figure 3.2 – Production of DMS in Presence or Absence of DMSO.

Log_{10} ppb of dimethyl sulfide (DMS) produced by *E. coli* B (ATCC) in the presence (1.25% v/v) or absence (0% v/v) of dimethyl sulfoxide (DMSO). *N*=3; bars showing standard error; asterisk indicates significance.

3.3.4 Influence of DMSO on antimicrobial testing

A comparison of results from two methods of MIC testing are shown in Table 3.2. Both methods produced the same MIC values for the oils tested. DMSO did not have a negative or beneficial effect when used as a solvent in antimicrobial testing.

Table 3.2 – Effect of DMSO on Antimicrobial Activity.

Comparison of MIC (% v/v) results for essential oils against *E. coli* B (ATCC 23848) determined using broth microdilution method and agar dilution method (*N=3*).

Essential Oil	Broth Microdilution MIC (% v/v)	Agar Dilution MIC (% v/v)
Cinnamon Bark	0.03	0.03
Clove	0.125	0.125
Tea tree	1.0	1.0

3.3.5 Disc diffusion assay

The results from the antimicrobial susceptibility screening assay demonstrated that all the EOs used in this study were antimicrobial against most, if not all, the bacteria tested (Table 3.3). Three of the EOs, cinnamon bark, clove and thyme, produced zones of inhibition against all strains tested, including *P. aeruginosa* PAO1, demonstrating broad spectrum activity. Inhibition zones shown by thyme and cinnamon bark EO were significantly larger ($P<0.05$) than that of the gentamicin antibiotic reference in 70% and 60% of the strains used, respectively. Thyme produced zones of inhibition ranging from 17.1 - 48.2 mm,

whilst cinnamon bark produced zones ranging from 16.7 – 36.2 mm. Rose geranium EO showed activity against all strains excluding *P. aeruginosa* NCTC 8505, producing zones ranging from 5.3 – 18.3 mm. Cinnamon leaf EO showed activity against all strains excluding *P. aeruginosa* PAO1, producing zones ranging from 8.7 – 18.2 mm. Lavender, lemongrass, lime, rosemary, rosewood and tea tree EOs showed effects against both Gram positive and Gram negative bacteria, excluding the *Pseudomonas* species. Of these oils, lemongrass produced the largest zones of inhibition, ranging from 10.1 - 36.9 mm. The smallest zones of inhibition were produced by bergamot, grapefruit, lemon, manuka and sweet orange EOs. Blank disc negative controls did not affect the growth of any of the strains. In accordance with the breakpoint tables for inhibition zone interpretation published by the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2018), all bacteria in this study were sensitive to gentamicin with the exception of MRSA and *A. baumannii* NCTC 12156.

3.3.6 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Nine EOs were selected, based on their antimicrobial activity shown in the disc diffusion assay, to determine their MIC and MBC (Table 3.4). The data shows that rose geranium, rosewood and tea tree EOs produced MICs between 0.5 and 4% or greater (v/v) against most strains and that MBC values for these are either equal or double the MIC value. Although Manuka EO demonstrated a MIC as low as 0.06% (v/v), the inhibitory effects were only against Gram-positive *S. aureus* strains. Manuka EO did not demonstrate any bactericidal

action at <4% (v/v). Thyme, lemongrass, cinnamon leaf and clove EOs presented MICs and MBCs of ≤1.0% (v/v) for all strains of bacteria excluding the *P. aeruginosa* strains. The only EO which demonstrated broad spectrum antimicrobial activity was cinnamon bark EO. All the bacteria tested including the *Pseudomonas* spp. were inhibited by cinnamon bark EO at ≤0.25% (v/v), which also demonstrated bactericidal activity at the same concentration.

Table 3.3 – Screening of Essential Oils.

Antimicrobial activity of fifteen essential oils using disc diffusion assay. Values are mean inhibition zone (mm) ± SE of 9 replicates (*N*=3).

Essential Oil	*E. coli* B ATCC 23848	*E. coli* NCTC 9001	*Staph. aureus* ATCC 6358	*Staph. aureus* NCTC 12981	Hospital Acquired MRSA isolate	MSSA NCTC 13297	*Ps. aeruginosa* PAO1	*Ps. aeruginosa* NCTC 8505	*Ac. baumannii* NCTC 12156	*Ac. baumannii* ATCC 17978
Bergamot	11.0±0.4†	-	7.0±0.0	10.7±0.4	6.4±1.6	5.0±1.3	-	-	5.1±1.3	4.7±1.2
Cinnamon Bark	36.2±1.2†	26.6±0.7†	30.8±0.5†	28.7±0.5	35.4±1.8†	27.7±1.1	17.3±0.7	16.7±1.4	28.6±0.7†	24.7±0.8†
Cinnamon Leaf	18.2±0.4	13.2±0.2	15.4±0.2	17.9±0.7	14.8±0.2	12.9±0.3	-	8.7±0.3	17.9±0.2	16.0±0.0
Clove	20.4±0.6	13.2±0.4	15.0±0.7	20.1±0.8	15.3±1.1	13.3±0.1	5.2±1.3	5.2±1.3	18.1±0.4†	15.8±0.5
Grapefruit	13.6±0.5	8.5±1.4	9.8±0.3	11.2±0.7	11.0±0.4	10.3±0.7	-	-	2.3±1.2	-
Lavender	12.3±0.7	7.7±0.3	14.7±1.7	12.1±0.4	11.6±1.0	9.8±0.2	-	-	8.9±0.5	9.1±0.3
Lemon	2.9±1.0	-	9.0±0.9	6.4±0.7	6.2±0.8	4.7±2.1	-	-	-	-
Lemongrass	32.6±1.0†	10.1±0.2	29.2±0.9	27.6±0.7	36.9±0.8†	26.8±2.1	-	-	14.2±1.5	24.6±2.6
Lime	14.7±0.8	10.6±1.1	11.1±0.3	12.8±0.6	10.1±0.8	10.1±0.7	-	-	12.0±0.6	11.6±0.2
Manuka	7.3±0.3	5.3±1.5	19.7±4.0	20.9±3.9	21.6±4.7	17.3±3.7	-	-	5.0±1.2	-
Sweet Orange	9.1±1.9	3.0±1.3	11.3±3.2	7.9±3.5	9.3±5.4	7.4±4.3	-	-	2.3±1.3	-
Rose Geranium	18.3±0.3	11.3±0.3	17.7±0.6	15.5±0.8	12.3±0.4	13.4±0.3	5.3±1.0	-	8.6±0.2	11.4±0.6
Rosemary	15.6±1.2	16.1±0.3	16.8±0.7	19.5±2.1	19.2±1.4†	17.4±1.1	-	-	16.5±1.3	14.7±1.4
Rosewood	21.7±2.0	14.7±0.7	16.4±2.1	20.3±1.0	16.2±0.4	14.0±0.7	-	-	20.04±1.1†	16.6±0.5
Tea Tree	16.7±0.6	16.4±0.6	23.4±1.7	22.4±2.1	17.2±0.5	13.5±1.0	-	-	18.9±1.2†	16.6±0.7
Thyme	46.2±4.8†	37.2±1.1†	48.2±2.1†	36.4±2.2	35.4±3.0†	35.8±2.8†	21.2±2.0	17.1±4.5	41.6±2.1†	36.4±5.3†
Gentamicin*	24.4±0.2	22.7±0.2	27.7±0.2	35.3±0.2	12.4±0.2	26.4±0.2	20.3±0.2	26.0±0.0	12.1±0.1	19.6±0.2

* gentamicin disc (30 µg)

- no activity

† value is significantly greater than gentamicin positive reference ($P<0.05$)

Table 3.4 – Inhibitory and Bactericidal Activity of Essential Oils.

The antimicrobial activity of eight essential oils, using the broth microdilution method to find minimum inhibitory concentration and minimum bactericidal concentration (% v/v) of 12 replicates (*N*=4).

Essential Oil	*E. coli B* ATCC 23848		*E. coli* NCTC 9001		*Staph. aureus* ATCC 6358		*Staph. aureus* NCTC 12981		Hospital Acquired MRSA isolate		MSSA NCTC 13297		*Ps. aeruginosa* PAO1		*Ps. aeruginosa* NCTC 8505		*Ac. baumannii* NCTC 12156		*Ac. baumannii* ATCC 17978	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Cinnamon Bark	0.03	0.125	0.06	0.125	0.06	0.125	0.03	0.06	0.015	0.015	0.03	0.125	0.125	0.125	0.25	0.25	0.03	0.125	0.06	0.125
Cinnamon Leaf	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.25	0.125	0.25	>4.0	>4.0	>4.0	>4.0	0.125	0.125	0.125	0.125
Clove	0.125	0.125	0.125	0.125	0.125	0.25	0.125	0.25	0.25	0.25	0.25	0.25	>4.0	>4.0	>4.0	>4.0	0.125	0.125	0.125	0.125
Lemongrass	0.25	0.25	1.0	1.0	0.25	0.25	0.25	0.25	0.12	0.25	0.25	0.25	>4.0	>4.0	>4.0	>4.0	1.0	1.0	0.5	1.0
Manuka	>4.0	>4.0	>4.0	>4.0	0.06	>4.0	0.06	>4.0	0.06	>4.0	0.06	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0
Rose Geranium	1.0	2.0	>4.0	>4.0	0.5	1.0	1.0	2.0	1.0	2.0	1.0	2.0	>4.0	>4.0	>4.0	>4.0	2.0	4.0	>4.0	>4.0
Rosewood	0.5	1.0	1.0	2.0	1.0	2.0	1.0	2.0	1.0	2.0	1.0	2.0	>4.0	>4.0	>4.0	>4.0	0.5	1.0	1.0	1.0
Tea tree	1.0	1.0	1.0	1.0	2.0	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0	4.0	4.0	2.0	2.0
Thyme	0.125	0.25	0.25	0.25	0.125	0.50	0.25	0.25	0.25	0.50	0.25	0.50	>4.0	>4.0	>4.0	>4.0	0.125	0.125	0.25	0.25

3.3.7 Gas chromatography mass spectrometry (GC-MS) analysis

Components of cinnamon bark and cinnamon leaf EO and their percentage composition are reported in Table 3.5. Cinnamon bark EO is composed of 85.312% Σ-cinnamaldehyde, whereas cinnamon leaf EO is 84.481% eugenol.

3.3.8 Time-kill assay

A time-kill assay was performed to evaluate the bactericidal potential of cinnamon bark EO against *P. aeruginosa* PAO1 (Figure 3.3). At the MIC, 0.125% (v/v), cinnamon bark EO was bactericidal (three-log reduction) against *P. aeruginosa* PAO1 after 6 h. At concentrations two-fold and four-fold of the MIC, 0.25% (v/v) and 0.5% (v/v), a 99.999% (five-log) kill, was achieved within 30 and 6 min respectively. At the higher EO concentrations of 1% and 2% (v/v), 5-log reduction was achieved within 2 min.

Table 3.5 – Analysis of Cinnamon Bark and Leaf Essential Oil

Percentage (%) composition of components within cinnamon bark and cinnamon leaf essential oil after gas chromatography mass spectrometry analysis.

Component	Percentage (%) of total composition of oil	
	Cinnamon Bark	**Cinnamon Leaf**
Σ-Cinnamaldehyde	85.312	0.185
D-limonene	4.665	-
Eucalyptol	3.823	0.160
Eugenol	3.366	84.481
Benzyl benzoate	1.935	2.752
β-linalool	0.897	1.627
Caryophyllene	-	3.224
Acetyl eugenol	-	2.191
α-pinene	-	0.982
Camphene	-	0.282
β-terpinene	-	0.242
α-phellandrene	-	0.957
3-carene	-	0.04
Isoterpinolene	-	0.062
m-cymene	-	0.638
2-thujene	-	0.201
p-menth-1-en-8-ol	-	0.122
Copaene	-	0.633
Cinnamyl alcohol acetate	-	0.640
α-caryophyllene	-	0.434
Caryophyllene oxide	-	0.141
Total	99.998	99.994

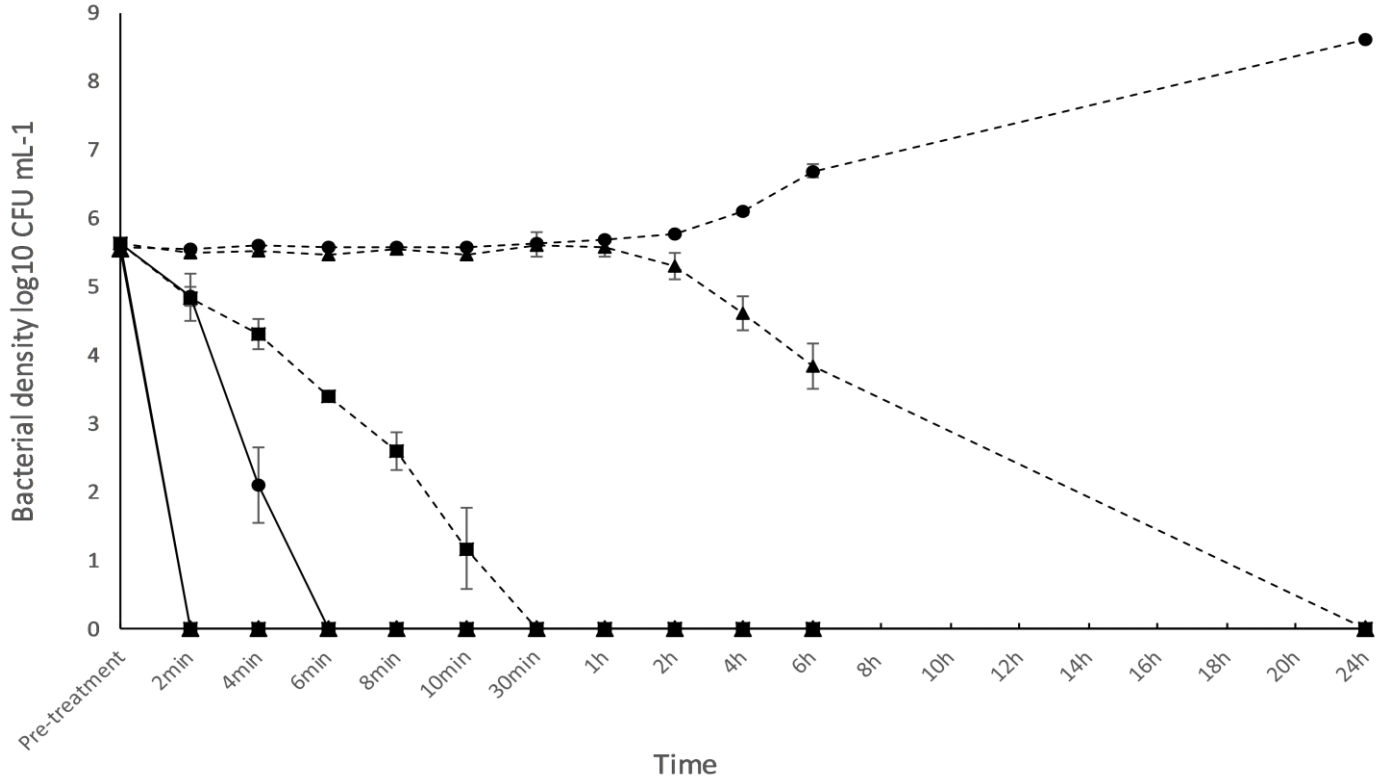


Figure 3.3 – Time Kill Assay

Time-kill of *P. aeruginosa* PAO1 in presence of cinnamon bark essential oil. Essential oil concentrations: 0% (v/v); 0.125% (v/v); 0.25% (v/v); 0.5% (v/v); 1.0% (v/v); 2.0% (v/v). Bars show standard error. *N*=11 for 0% EO control sample, *N*=3 for remaining samples

3.3.9 Metabolic activity assay

Reduction of TTC from a yellow to red colour was observed in microtitre wells containing viable *P. aeruginosa,* but no colour change was observed when cells were non-viable (Figure 3.4). Negative control wells with TSB containing no bacteria remained clear with no colour change. *P. aeruginosa* continued to show metabolic activity after 10 min of contact time when cinnamon bark EO was at 0%, 0.125% and 0.25% (v/v), with some activity after 4 min contact time with EO at 0.5% (v/v), but no activity after 6 min. Metabolic activity had ceased after 2 min of contact time at concentrations equal or greater to 1% (v/v).

3.3.10 Scanning electron microscopy (SEM)

Electron micrographs of both untreated and cinnamon bark EO treated *P. aeruginosa* cells are presented in Figures 4.5-4.8. The untreated cells (
Figure **3.5** and Figure 3.6) show a turgid structure with a particulate surface and are of expected lengths (1 to 5 µm). In contrast, cells treated with cinnamon bark EO showed an altered morphology (Figure 3.7 and
Figure 3.8) with cells appearing collapsed with loss of turgidity and few surface particles after 10 min contact time with 2% (v/v) cinnamon bark EO.

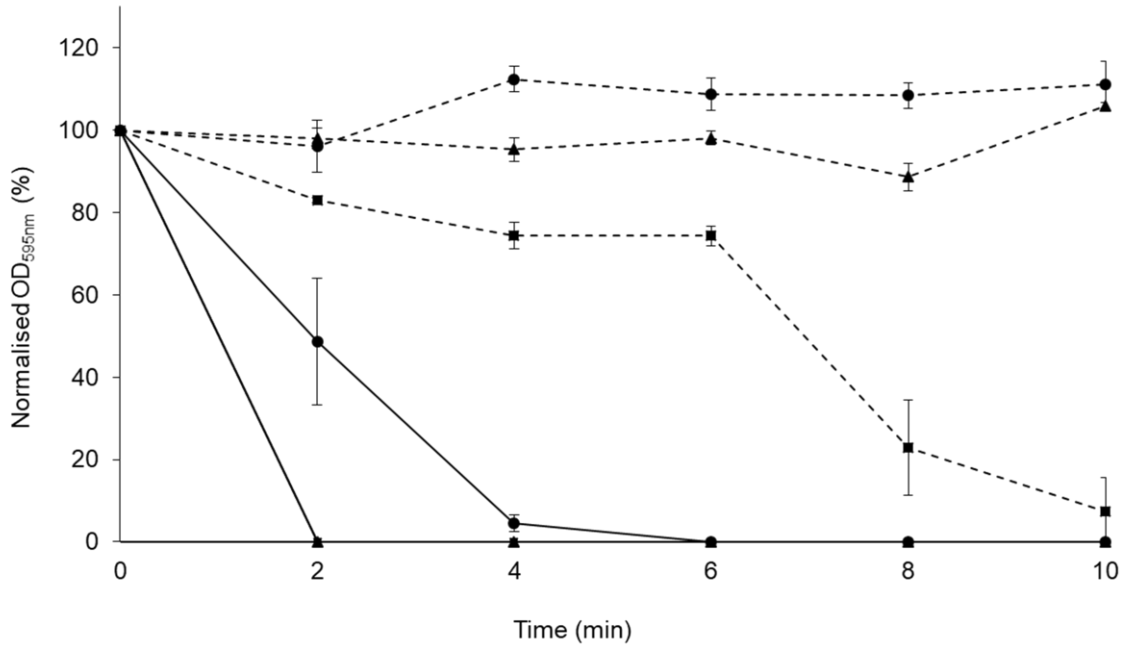


Figure 3.4 – Metabolic Activity Assay

Optical density (OD595nm) data for triphenyltetrazolium chloride metabolic activity assay after treating *P. aeruginosa* PAO1 with cinnamon bark essential oil, normalised to T0 and expressed as a percentage. Essential oil concentrations: 0% (v/v); 0.125% (v/v); 0.25% (v/v); 0.5% (v/v); 1.0% (v/v); 2.0% (v/v). Bars show standard error (*N*=9).

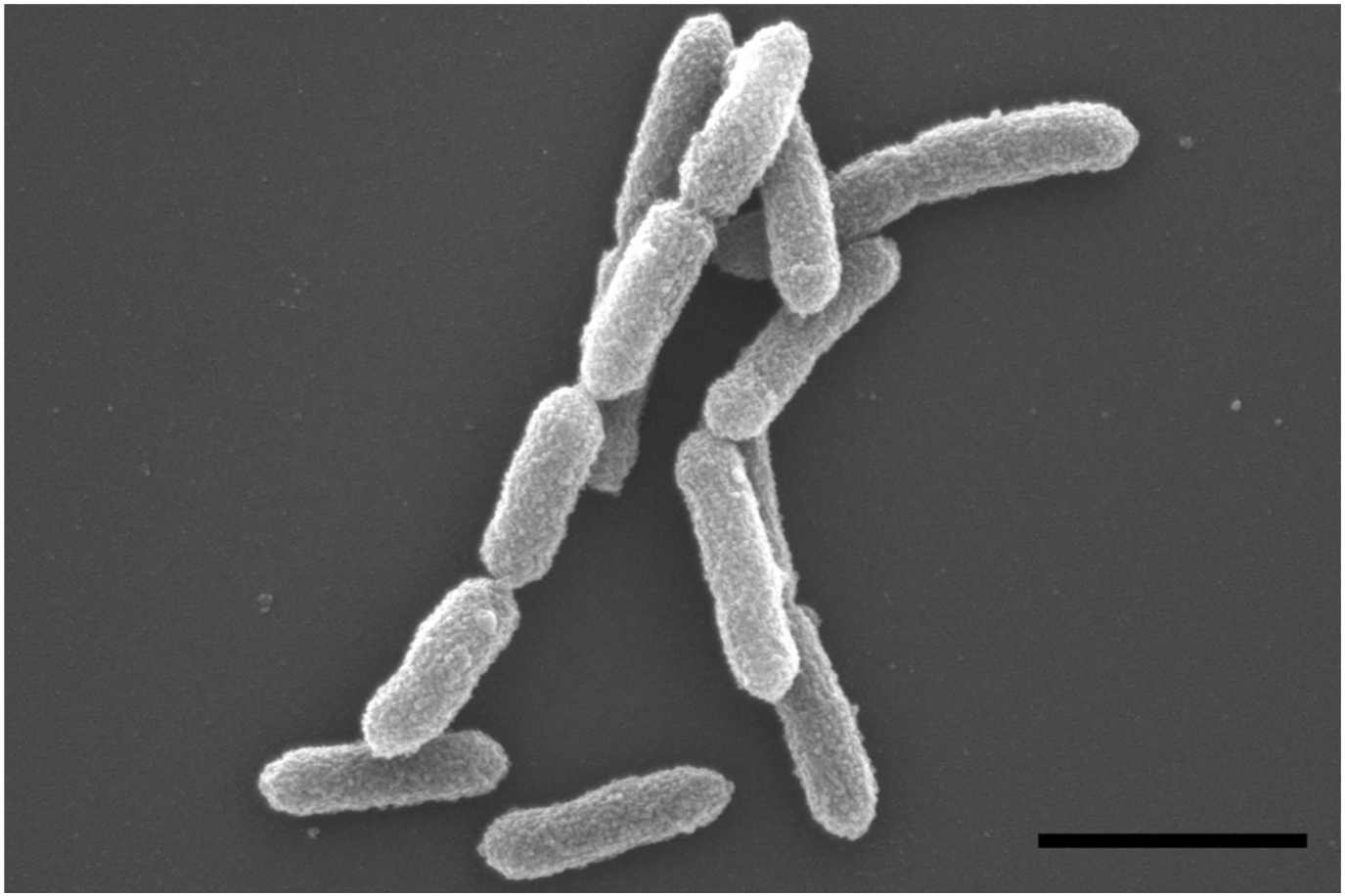


Figure 3.5 – Untreated *P. aeruginosa.*

Scanning electron micrograph showing untreated *P. aeruginosa* PAO1 cells. Scale bar two µm

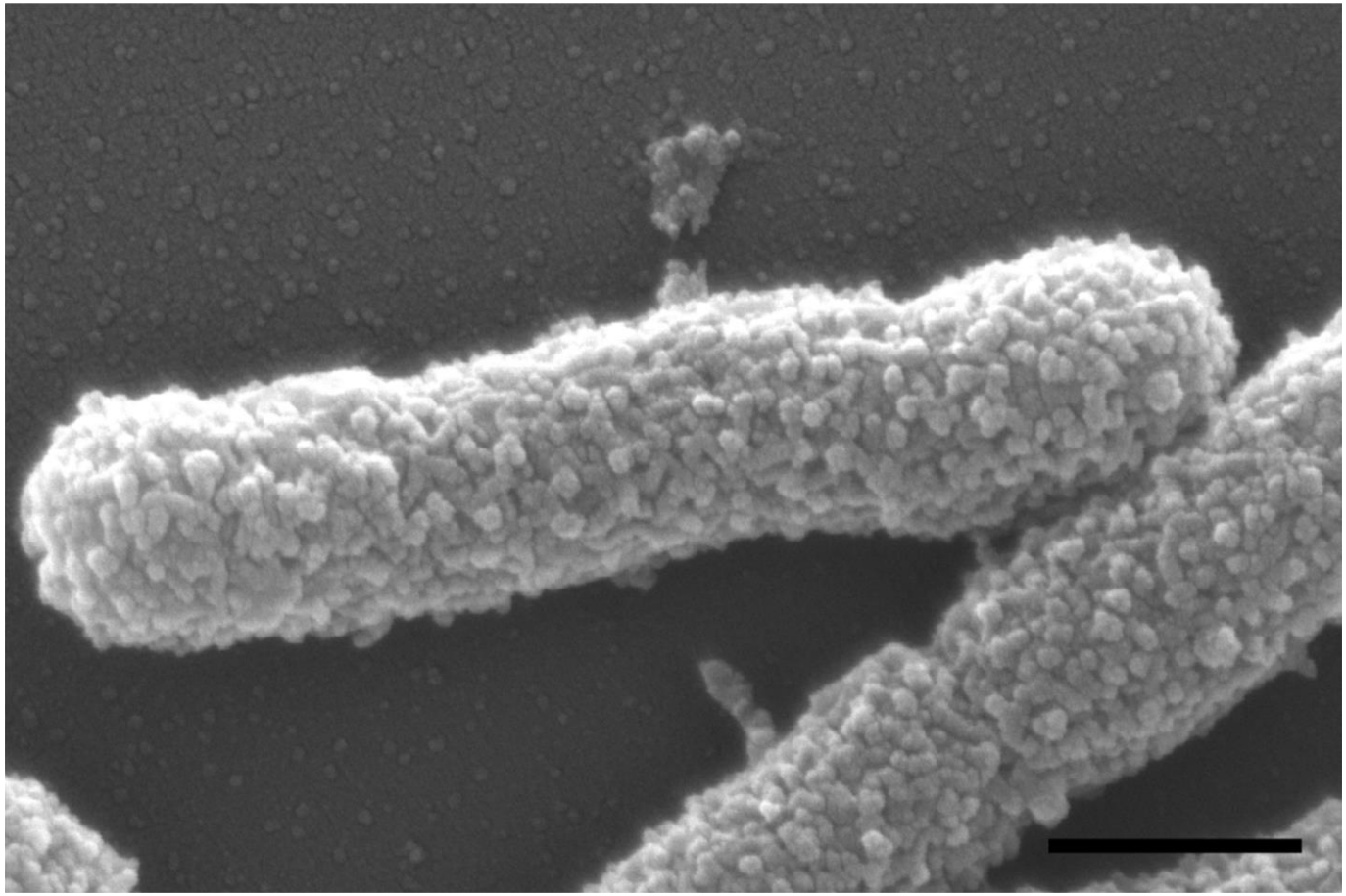


Figure 3.6 – Untreated *P. aeruginosa.*

Scanning electron micrograph showing untreated *P. aeruginosa* PAO1 cells. Scale bar 500 nm

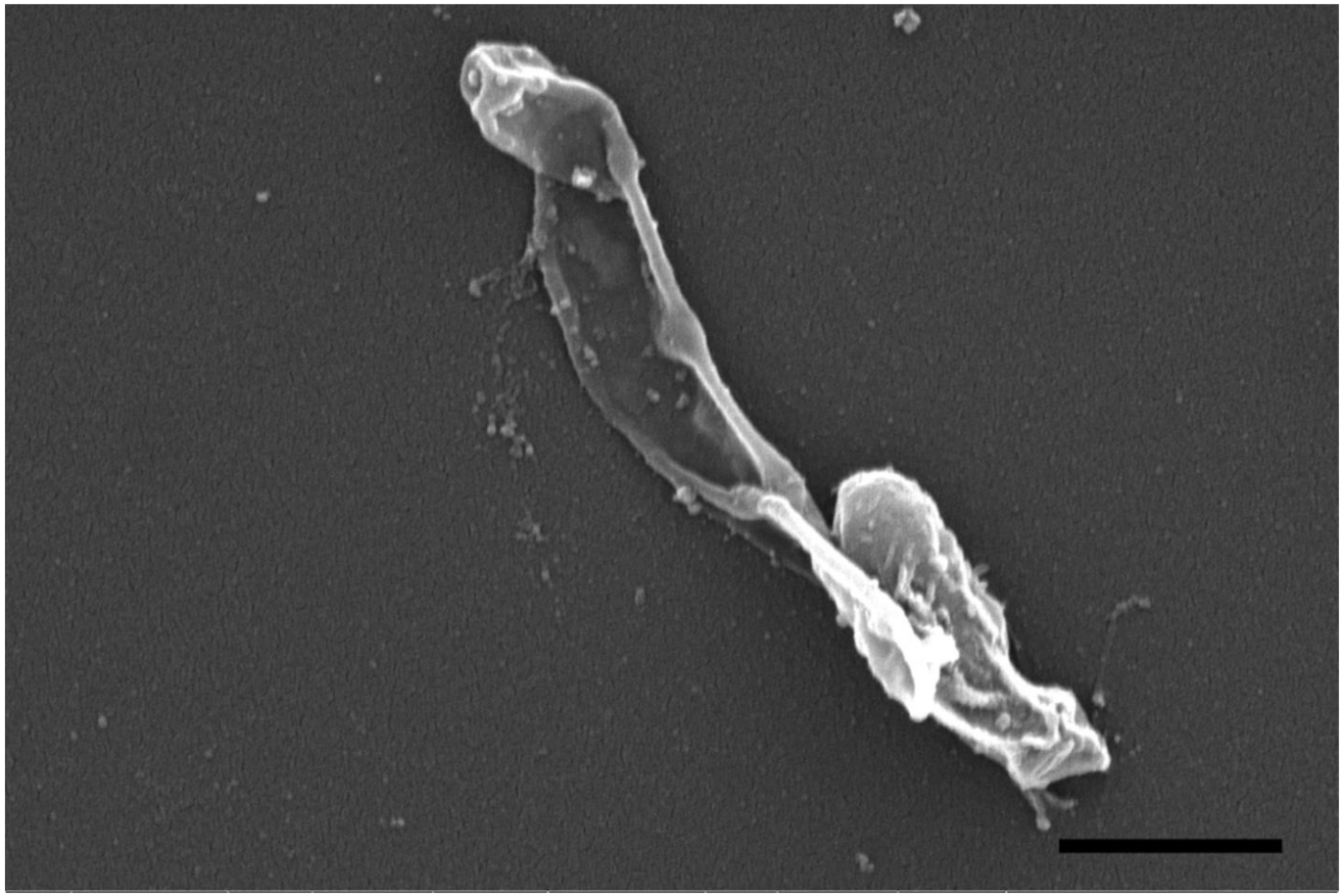


Figure 3.7 – Treated *P. aeruginosa.*

Scanning electron micrograph showing *P. aeruginosa* PAO1 cells treated with 2% cinnamon bark EO for 10 min. Scale bar one µm.

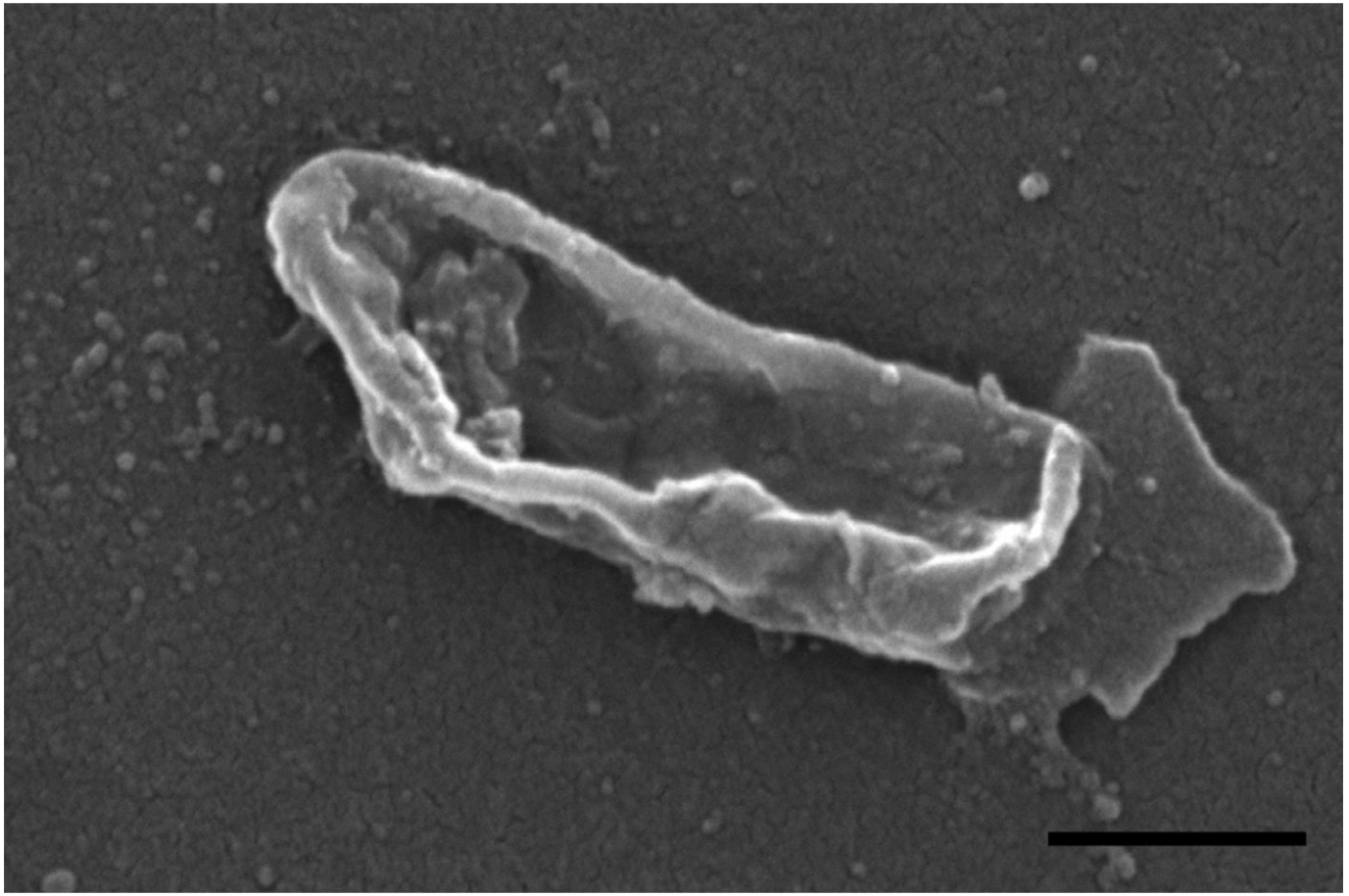


Figure 3.8 - Treated *P. aeruginosa.*

Scanning electron micrograph showing *P. aeruginosa* PAO1 cells treated with 2% cinnamon bark EO for 10 min. Scale bar 500 nm.

3.3.11 Release of nucleic acids and proteins

Relative optical densities of filtrate from *P. aeruginosa* untreated or treated with cinnamon bark EO measured at 260 nm and 280 nm are displayed in Figure 3.9 and Figure 3.10, respectively. Increased $OD_{260\ nm}$ readings signifies an increase in nucleic acids, whilst an increase in $OD_{280\ nm}$ indicates an increase in proteins (Miksusanti *et al.* 2008) released from bacterial cells. An increase was seen in both $OD_{260\ nm}$ and $OD_{280\ nm}$ when bacteria were treated with cinnamon bark EO at either 0.125% or 2% (v/v) when compared to the untreated cultures. Release of nucleic acids was significantly different after treatment with 0.125% (v/v) EO ($P<0.05$) and 2% (v/v) EO ($P<0.01$) when compared to untreated bacteria.

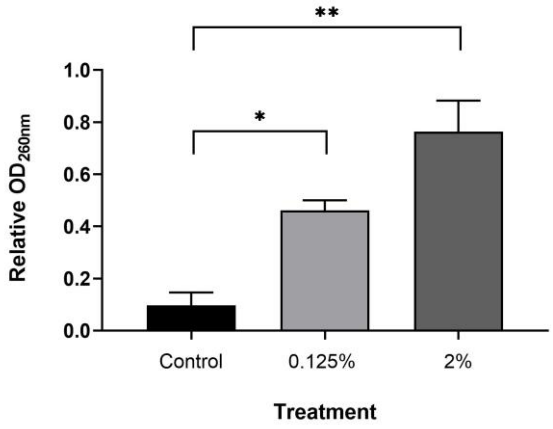


Figure 3.9 – Nucleic Acid Release Assay

Relative OD_{260nm} of filtrate from *P. aeruginosa* control culture, after treatment with cinnamon bark EO at 0.125% and 2% (v/v). Control = no EO present; *N*=3; bars showing standard error; asterisk indicates significance.

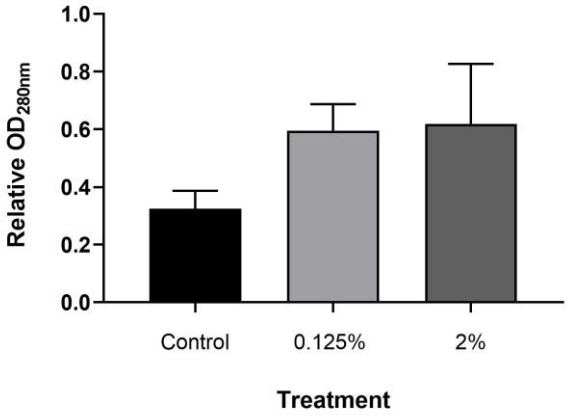


Figure 3.10 – Protein Release Assay

Relative OD_{280nm} of filtrate from *P. aeruginosa* control culture, after treatment with cinnamon bark EO at 0.125% and 2% (v/v). Control = no EO present; *N*=3; bars showing standard error.

3.4 Discussion

When searching for novel antimicrobials, several different methods can be employed to establish the MIC value of an antimicrobial, with more common techniques including broth microdilution. When using essential oils (EOs) as an antimicrobial, their hydrophobicity often poses a problem when trying to disperse them in solutions for dilution. A solvent is often used in antimicrobial screening to assist with the dilution of insoluble compounds, and DMSO is a frequently chosen solvent in broth dilution methods (Ugur *et al.* 2016, Oliveira *et al.* 2017, Brahmbhatt *et al.* 2018, Romulo *et al.* 2018). The effects of solvents on bacterial growth have been investigated in other studies (Wadhwani *et al.* 2012), which found DMSO to be the best solvent when used at concentrations of 3% or less compared to methanol or ethanol. The suitability of DMSO amongst the Gram-negative and Gram-positive bacteria used in this study was assessed. DMSO when used at concentrations <2.5% (v/v) had little to no effect on the growth of any organism, with the exception of *E. coli* B (ATCC 23848), which is similar to the findings previously seen in the study by Wadhwani *et al*. (2012). However, in the present study, *E. coli* B (ATCC 23848) in the presence of either concentration of DMSO showed a promotion of growth, double that of the control, and produced an unpleasant odour after overnight incubation. Similar results were reported by Markarian *et al.* (2002) who found that the growth rate of *E. coli* in the presence of DMSO (1-2% concentration) was almost 4-fold higher than the control. However, there was no report of an emitted odour. Following headspace analysis using SIFT-MS, it was shown that when *E. coli* was grown in the presence DMSO, DMS was produced at levels 100-fold greater than if grown without. DMS is an

organosulphur compound that is ubiquitous in marine environments and released by phytoplankton and algae (Todd *et al.* 2010). DMS is also known to have an unpleasant odour (Siebert *et al.* 2010). DMS has been shown to be a product of *E. coli* in studies looking at DMS production in co-cultured blood samples (Umber *et al.* 2013), and in studies of volatile organic compounds produced by inoculated urine samples (Storer *et al.* 2011). Umber *et al*. (2013) speculate that this DMS production could be a stress related response of *E. coli* in the presence of bactericidal components. However, data from Storer *et al*. (2011), coupled with findings from the present study, suggest that DMS is produced without the presence of an antimicrobial compound. Many microorganisms use DMSO as a terminal electron acceptor, ultimately reducing the compound to DMS through use of a DMSO reductase (De Bont *et al.* 1981), and it has been reported that *E. coli* possesses several of these DMSO reductase enzymes (Sambasivarao *et al.* 1991).

Due to the increased growth exhibited by *E. coli* in the presence of DMSO, further investigations were carried out to assess whether DMSO affected EO MIC values against this strain. This was determined by the use of an alternative method of antimicrobial testing, the agar dilution method, that did not use a solvent. It was established that the presence of DMSO did not have an effect on results of antimicrobial testing, thus, DMSO was used throughout this thesis as a solvent to aid in the homogeneity of essential oils in solutions.

This chapter continued to examine the effect of EOs against pathogenic bacteria, some of which exhibit antimicrobial resistance. Disc diffusion assays using EOs at 100% (v/v) concentration demonstrated antimicrobial activity against a selected the panel of Gram-positive and Gram-negative bacteria and

proved to be a useful screening method to identify EOs that showed antimicrobial activity against these organisms. The use of an established antibiotic (gentamicin) provided a reference against which the sensitivity of the bacteria was assessed, and EUCAST breakpoint tables were used to determine expected zones of inhibition (EUCAST 2018). Gentamicin sensitivity was observed in all tested bacteria with the exception of MRSA and *A. baumannii* NCTC 12156. Zones of inhibition breakpoints conferring resistance for MRSA and *A. baumannii* treated with gentamicin were >18 mm and >17 mm, respectively.

Following the disc diffusion assay the greatest inhibitory activity was shown following exposure to thyme, cinnamon bark and lemongrass. Of these, thyme EO was most effective, an observation reported previously by Semeniuc *et al.* (2017) where it was most effective when compared to three other oils. Although the MRSA strain used was resistant to gentamicin in this study, cinnamon bark, lemongrass, manuka, rosemary and thyme EOs all produced zones greater than 18 mm, exceeding the resistance breakpoint of gentamicin against this pathogen. *A. baumannii* NCTC 12156 also showed resistance against gentamicin, although tea tree, thyme, rosewood, clove, cinnamon bark and cinnamon leaf EOs all produced zones ≥ 17 mm, exceeding the resistance breakpoint of gentamicin against this bacteria. These data are in line with published reports (Doran *et al.* 2009, Adukwu *et al.* 2012, Priti and Shridhar 2012, Yap *et al.* 2014, Sakkas *et al.* 2016), which indicate that EOs are capable of working effectively against bacteria resistant to commercial antibiotics. However, as pointed out by Jorgensen and Ferraro (2009), whilst the disc diffusion method has a place in routine or preliminary testing, the

results should be considered more qualitative than quantitative. Thus, further testing was carried out to assess the minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) of selected oils which produced the largest zones of inhibition. MIC and MBC testing showed that lemon, lemongrass, manuka, sweet orange and tea tree EO were more effective against the Gram-positive bacteria in comparison to the Gram-negative bacteria. Lemongrass and manuka in particular were shown to have lower MICs/MBCs for *S. aureus* when compared to other bacteria tested. These findings are in agreement with studies that found manuka EO to be more effective against *S. aureus* compared to *E. coli* and *P. aeruginosa* (Porter and Wilkins 1998), and studies where lemongrass EO also showed good effect against Gram-positive bacteria (Hammer *et al.* 1999). In the present study, cinnamon bark at very low concentrations (i.e. 0.015% v/v), was bactericidal against MRSA, which is supported by Cui *et al.* (2016) where cinnamon oil was found to be bactericidal against MRSA at 0.25 mg mL^{-1}, approximately 0.025% (v/v).

Cinnamon leaf, clove, lemongrass, rosewood and thyme oils at <4% (v/v) were bactericidal against all the bacteria, excluding *Pseudomonas* species. This is in agreement with a study by Kavanaugh and Ribbeck (2012), who reported that clove, thyme and tea tree EO were not bactericidal against *Pseudomonas* spp. at concentrations less than or equal to 4% (v/v).

Other studies have shown that EOs are more effective against Gram-positive bacteria (Lodhia *et al.* 2009), which is supported by some of the results presented here. However, equal or greater efficacy was observed when the Gram-negative bacteria were treated with some of the oils. Bergamot, cinnamon bark, cinnamon leaf, clove, grapefruit, lime, rose geranium and

rosewood EOs all produced greater zones of inhibition in some Gram-negative bacteria compared to zones produced for Gram-positive bacteria. Of the oils tested for MIC, cinnamon leaf, clove, rosewood, tea tree and thyme had lower or equal MIC for Gram-negative bacteria. Cinnamon bark EO demonstrated bactericidal effects against all the strains used in this study at very low concentrations (i.e. ≤ 0.25% v/v).

When comparing the oils to justify further testing, disc diffusion testing indicated that cinnamon bark, lemongrass and thyme were most effective overall, exhibiting the largest zones of inhibition on average. When looking at MIC and MBC testing, again cinnamon bark and thyme were similar in their efficacy. However, whilst both cinnamon bark and thyme exhibited broad spectrum activity and efficacy at low concentrations, only cinnamon bark EO was effective against *Pseudomonas* spp. at concentrations of <4% (v/v). Therefore, cinnamon bark EO was selected for further investigations to identify its capability of rapid bactericidal activity and its possible mode of action.

GC-MS analysis showed that the major component of cinnamon leaf oil used in this study was eugenol, which was 84.5% of the total oil composition, a similar finding to other published studies (Mallavarapu *et al.* 1995, Paranagama *et al.* 2001, Raina *et al.* 2001, Jayawardena and Smith 2010). In contrast, the major component of the cinnamon bark oil used in this study was Σ-cinnamaldehyde at 85.3% of the total composition. The high levels of this component in the bark EO has been demonstrated elsewhere in other studies (Paranagama *et al.* 2001, Jayawardena and Smith 2010, Shahina *et al.* 2018). This contrasting composition of the two oils provides an explanation of their different effects as antimicrobials, as it has been shown that cinnamaldehyde

has a superior antimicrobial effect when compared to eugenol (López *et al.* 2007, Sanla-Ead *et al.* 2011, Brnawi *et al.* 2018). The differences seen in oil composition also highlight the importance of adequate analysis of EOs, using methods such as GC-MS, to confirm the origin of the oil and identify which part of the plant it is derived from. This is especially important in the instance that oils supplied are incorrectly identified by the supplier, as is what happened with the oils described in section 2.3.

From the time-kill assay conducted in this study, cinnamon bark EO exhibited bactericidal effects with rapid killing action (three-log reduction) at ≤30 min at concentrations ≥0.25% (v/v). The TTC assay was used to indicate the presence or absence of metabolically active cells which would convert TTC to a coloured formazan derivative (Sabaeifard *et al.* 2014). The results obtained correlate well with the time-kill assay, however, it has been reported that TTC reduction may not correlate exactly with MIC, and end-points are less easily determined using the TTC assay when compared to that of other redox indicators, such as resazurin (Mann and Markham 1998).

The bactericidal effect of cinnamon bark EO on *P. aeruginosa* cell morphology was examined by scanning electron microscopy (SEM). After only 10 min of exposure to the EO, morphological changes to the treated cells were pronounced, and suggested total cell collapse, loss of turgor and likely loss of viability. Reports on the mechanism of action of EOs suggest their role in the destruction of the cell wall, damage to the cytoplasmic membrane and membrane proteins, and cell leakage (Goldbeck *et al.* 2014). This suggestion is supported by data from the membrane permeability assay, which indicate that cinnamon bark EO causes leakage of nucleic acids, a probable result of

a compromised cell membrane. This is in agreement with Bouhdid *et al.* (2010) who found that cinnamon bark oil affected the membrane of *P. aeruginosa* which ultimately led to cell death. This contrasts however, with the findings by Cox and Markham (2007) and Helander *et al.* (2000) who showed that Σ-cinnamaldehyde, a predominant component of the cinnamon bark EO used in this study, did not have an effect on the membrane of *P. aeruginosa* and was deemed to not be a pore forming compound. This highlights the importance of using whole oils, and not their isolated compounds, as it may not reflect the antimicrobial capability of an oil. It also further highlights the need for oil analysis to identify the blend of different compounds present and the role they play in the antimicrobial effects of an EO.

Cinnamon EO is sourced from either the bark or the leaf of the plants (Park *et al.* 2018), with the EO extracted from bark most often investigated for its antimicrobial activity (Nabavi *et al.* 2015). Cinnamon bark EO is already well described for its antimicrobial activity (Manso *et al.* 2014, Wu *et al.* 2015, Wen *et al.* 2016, Zhang *et al.* 2016, Chouhan *et al.* 2017), and shown to be highly bactericidal against *Pseudomonas* spp. (Utchariyakiat *et al.* 2016). However, to our knowledge, the present study is the first to demonstrate the rapid killing effect of the oil against *P. aeruginosa,* with contact times of <30 min.

Although 4% (v/v) was the highest concentration tested, and the maximum concentration recommended by the manufacturer for topical use, cinnamon oil is widely reported to be “Generally Regarded/Recognised as Safe” (GRAS) (Tzortzakis 2008, Xing *et al.* 2011, Ojagh *et al.* 2014, Cui *et al.* 2016, Bravo Cadena *et al.* 2018). As of May 2019, the Food and Drug Administration (FDA) consider cinnamon bark EO, from either *C. zeylanicum, C. cassia* or *C. loureirii*

to be GRAS (FDA 2019). A study by Adams *et al.* (2004) further supported the GRAS status of cinnamon oil after investigating the toxicity of 55 cinnamyl derivatives commonly found in cinnamon oil, used as flavouring agents, and determining them to be GRAS. Whilst toxicity testing for cinnamon oil is limited, Ranasinghe *et al.* (2012) report that preclinical *in vivo* studies are yet to show significant toxic effects but acknowledge that more clinical trials are needed to further understand the therapeutic safety of cinnamon in humans. Also, many of the studies that investigate toxicity of “cinnamon” in mouse or rat models are in fact testing the pure cinnamaldehyde component (Jenner *et al.* 1964, Hébert *et al.* 1994, Adams *et al.* 2004). However, cinnamon oil is recognised as a skin irritant (Veal 1996). Several case reports of contact dermatitis were described by Connolly *et al.* (2017), although patients in this report were subsequently patch tested for allergies and were all confirmed to be sensitive to cinnamaldehyde or other related compounds. A study by Bickers *et al.* (2005) reported that cinnamaldehyde below a concentration of 1.25% will not cause primary irritation, though it will be an irritant at concentrations greater than 3%. With these reports in mind, safety regarding the use of these natural compounds is a potential issue and risks must be recognised when contact with humans (and other animals) occurs. However, as previously described, antimicrobial effects of cinnamon bark oil seen in this study have been evident at concentrations as low as 0.015% (v/v) and effective concentrations throughout MIC testing do not exceed 0.25% (v/v). Furthermore, the intended use of the EO reported in this study is not directly associated with oral or dermal contact in humans. Contact would be the same as with any other antimicrobial disinfectant or sanitiser, and so toxicity of cinnamon EO does not

pose the same risks as those reported in case studies regarding adverse reactions (Bickers *et al.* 2005).

EOs have thus far shown their potential as antimicrobials, though their real-world application is not limited to this function. EOs have been used as antimicrobials, flavourings and fragrances in aromatherapy, medicine, industrial food and drink production and the cosmetic industry (Baser and Buchbauer 2015).

In summary, the preliminary experiments investigating DMSO highlighted the importance of investigating solvents that are used to disperse EOs, which may have an effect on results during antimicrobial screening. Growth promotion in antimicrobial testing may lead to inoculum effect, causing significant increases in MIC (Brook 1989), which would be detrimental to novel antimicrobial screening, however in this instance DMSO did not have an effect on MIC values. This chapter has also investigated the possible volatile organic compounds that microorganisms may produce in the presence of solvents. Further investigation of these compounds in the headspace of cultures supplemented with solvents, and additional work to fully refine the SIFT-MS analysis, is required. However, despite these limitations, this study highlights the potential of this technique for the rapid identification of volatile compounds in microbial cultures.

This chapter has shown that EOs are effective antimicrobial agents and many possess broad-spectrum activity. Cinnamon bark EO in particular has demonstrated strong bacteriostatic and bactericidal action against *P. aeruginosa*. This provides a sound basis for further work to explore the use of

cinnamon bark EO as an alternative to conventional antimicrobial products owing to its fast-acting bactericidal properties at low concentrations.

The work presents in this chapter has confirmed that cinnamon bark is an antimicrobial EO which provides broad spectrum, high bactericidal activity when used at low concentrations and within a short contact time. These qualities make it an ideal candidate as an alternative to current antimicrobials, for example on surfaces in clinical and food preparation applications, in disinfection and infection control. Many studies investigating time kill and bactericidal activity of EOs expose bacteria to longer contact times, though it is important that shorter contact times are investigated, and this study highlights the importance of performing antimicrobial tests using contact times <10 min. Findings from this study further support the reports that the mode of action of cinnamon bark EO against *P. aeruginosa* is due to effects on the membrane of the bacteria, leading to cell leakage and cell death, shown here by of SEM and membrane permeability assays. Although EOs are generally recognised as safe (GRAS) by the Food and Drugs Administration (FDA), including that of cinnamon (Maisanaba *et al.* 2017), more investigation is needed to assess cinnamon EO's application to control bacterial pathogens and subsequent interaction with humans. This study also highlights the importance of analysing these natural products before use, in order to determine their composition and to identify their key components. The composition and antimicrobial diversity observed amongst oils originating from different parts of the same species of plant emphasises the need for batch control and consistency when developing these oils for antimicrobial purposes.

CHAPTER 4

4 GROWTH OF BACTERIAL BIOFILMS AND TREATMENT OF *PSEUDOMONAS AERUGINOSA* BIOFILMS WITH CINNAMON BARK EO IN LIQUID PHASE

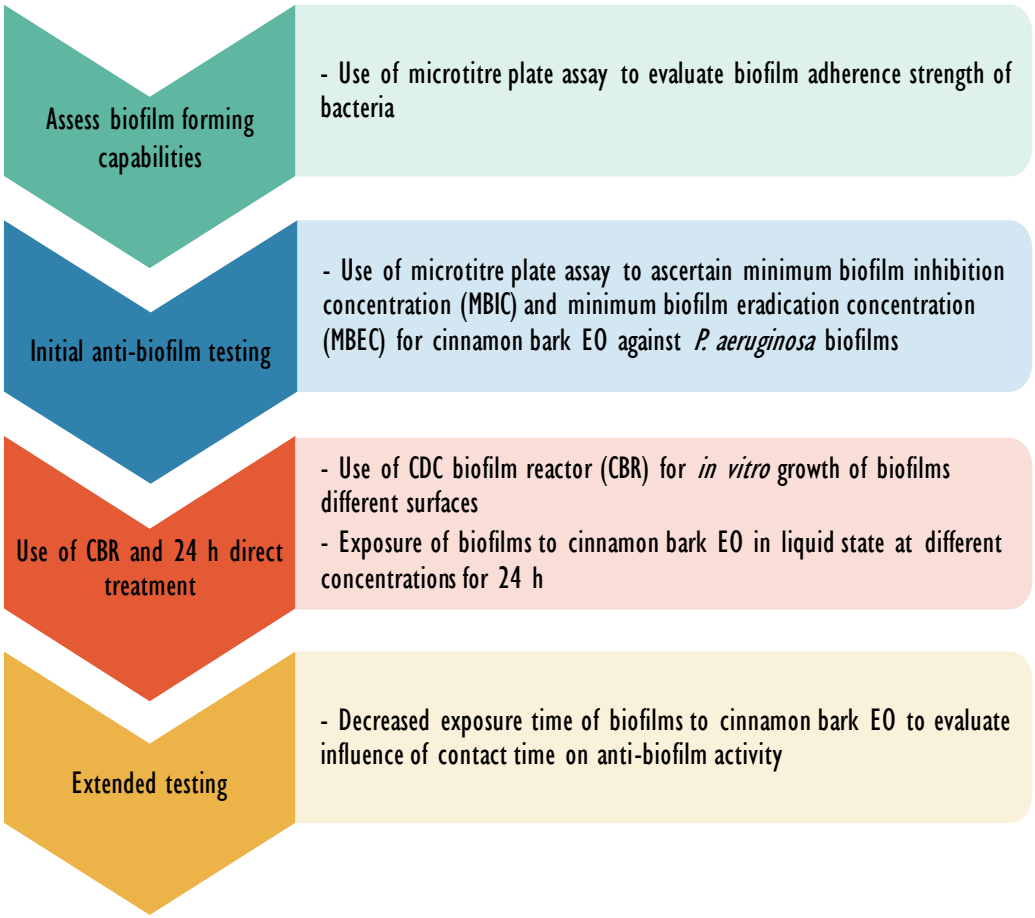
4.1 Introduction

An important feature of *Pseudomonas* is its ability to form and exist as robust, protective biofilms which aids in its survival and resistance to antimicrobials (Sabaeifard *et al.* 2014). Biofilm formation is often thought to be the primary underlying cause for the failure of an antimicrobial agent, with 65-80% of all infections believed to be biofilm-related (Coenye and Nelis 2010). Cells existing as a biofilm often have an increased resistance to antimicrobial agents of up to 10–1000 times, with multiple mechanisms of resistance recognised within a single community (Mah and O'Toole 2001). The resistance to antimicrobials shown by bacteria in biofilms can be explained by the failure of the antimicrobial agent to penetrate the biofilm, largely due to the presence of an exopolysaccharide matrix (EPS) that is secreted when the biofilm is maturing (Butt and Khan 2015). Difficulty to treat biofilms, their increased resistance and their significance in current infections, indicates a necessity to develop new ways to combat them. The antimicrobial activity of many EOs, including that of cinnamon EO against *Pseudomonas* spp., has been investigated previously and some studies, including this thesis, have shown that cinnamon bark EO has good antibacterial activity (Bouhdid *et al.* 2010, Utchariyakiat *et al.* 2016). However, these studies did not assess the effects of cinnamon bark EO against *Pseudomonas* biofilms. Where the effect of EOs on biofilms have been studied using alternative EOs and biofilm bacteria (Kalia

et al. 2015, Kim *et al.* 2015), different concentrations and contact times typically have not been investigated. Thus, the aims of experiments reported in this chapter were to elucidate the anti-biofilm effects of cinnamon bark EO in liquid state, against *P. aeruginosa* biofilms grown on different surfaces, and to establish an optimum concentration and contact time.

4.1.1 Chapter progression

The following flow diagram depicts a summary of the progression of this chapter.



4.2 Methods

4.2.1 Crystal violet (CV) biofilm assay

CV biofilm assay was carried out as described in section 2.4.1.

4.2.2 Minimum biofilm inhibition concentration (MBIC)

Methods to assess MBIC concentration were adapted from Stepanović *et al.* (2000) and Adukwu *et al*. (2012) and were similar to methods described in section 2.4.1. An overnight culture of *P. aeruginosa* was standardised to ~1.5 x 10^8 CFU mL^{-1} as described in section 2.2, with the exception of being diluted with TSB medium supplemented with 1% (v/v) glucose. One hundred microliters of this suspension were added to each well of a 96-well microtitre plate, in addition to 100 µL of TSB containing cinnamon bark EO concentrations, ranging from 0.015% to 2% (v/v) (prepared as described in section 2.3.2). Negative controls contained no EO. Plates were incubated at 37°C for 24 h. Then each well was carefully aspirated and washed three times using 250 µl of sterile phosphate buffered saline (PBS). Bacteria were fixed using 200 µL of methanol for 15 min, wells were emptied and then air dried. After air drying, wells were stained with 250 µL of 0.1% crystal violet for 5 min. Excess stain was removed by rinsing with tap water. After leaving to air dry, the bound stain was solubilised with 250 µl of 33% (v/v) acetic acid. The optical density (OD) of wells in the plate was then read using a TECAN Infinite® 200 PRO plate reader at 595 nm. Absorbance values of oil dilutions were subtracted from OD values to correct for turbidity exhibited by oils. Inhibition of biofilm growth was indicated if the OD was less than or equal to the OD cut-off (ODc), defined as three standard deviations above the mean OD of the

negative control. Three wells were used per EO concentration and the experiment was independently carried out in triplicate.

4.2.3 Minimum biofilm eradication concentration (MBEC)

Methods for assessing MBEC were adapted from Malic *et al.* (2013). Briefly, wells of a 24-well microtitre plate were filled with 1 mL of overnight culture that was standardised to ~1.5 x 10^8 CFU mL^{-1}, as described in section 2.2, with the exception of being diluted with TSB medium supplemented with 1% (v/v) glucose. Plates were incubated at 37°C for 24 h. Next, the medium was removed gently using a pipette and wells were washed with 1 mL of PBS. EO dilutions were prepared as described in section 2.3.2 and 1 mL of each EO was added to separate biofilms wells. Plates were incubated for a further 24 h at 37°C. The medium again was removed gently by aspiration and wells were washed with PBS. One millilitre of TSB was added to each biofilm, followed by disruption via repeated agitation with a pipette. Aliquots (100 µL) of each well were plated onto MHA in triplicate and incubated at 37°C for 24 h and observed for growth. The same 24 well plates were incubated for a further 6 h under the same conditions as before and aliquots were removed and plated after this time, to assess bacterial viability. MBEC was defined as the lowest concentration of EO capable of preventing regrowth after subsequent 6 h incubation step. Three independent experiments were carried out in triplicate.

4.2.4 Growth of *P. aeruginosa* biofilms in the Centre for Disease Control biofilm reactor (CBR)

Standardised 48 h mature *P. aeruginosa* PAO1 biofilms were grown on polycarbonate (PC) or stainless steel 316L (SS) coupons using the CBR as described in section 2.4.2.

4.2.5 Essential oil

Cinnamon bark EO was used as described in section 2.3.

4.2.6 Direct treatment of biofilms grown in CBR

A schematic of this method is demonstrated in Figure 4.1. Dilutions of cinnamon EO were made in 30 mL TSB to achieve concentrations of 2%, 0.2% and 0.02% (v/v), with DMSO used as a solvent at concentrations of 1.25%, 0.125% and 0.0125% (v/v), respectively. A control was used for each treatment with only TSB and DMSO at respective concentrations. Rods from the CBR holding mature biofilms, on either PC or SS coupons were removed from the reactor, rinsed with sterile phosphate buffered saline (PBS) and placed into the TSB containing cinnamon bark EO, previously described. Rods were removed from the treatment after 24 h of contact. In some cases, further testing at 5, 10, 30, 60 min and 6 and 12 h was carried out. After removal from treatment, rods were rinsed in sterile PBS and each coupon released into 10 mL of TSB, sonicated for 10 min and vortexed for 30 s. The disaggregated biofilms were diluted serially and plated onto MHA and incubated at 37°C for 24 h. The CFU mL^{-1} from three coupons were enumerated for each condition per experiment and the experiment was repeated in triplicate. Results were

expressed as mean log CFU mL^{-1}. When 10^{-0} dilution was plated, limit of detection was 10 CFU mL^{-1}.

4.2.7 Data analysis

Two-Way ANOVA and comparison of column factors was used to assess significant differences between treatments and controls following direct treatment of biofilms.

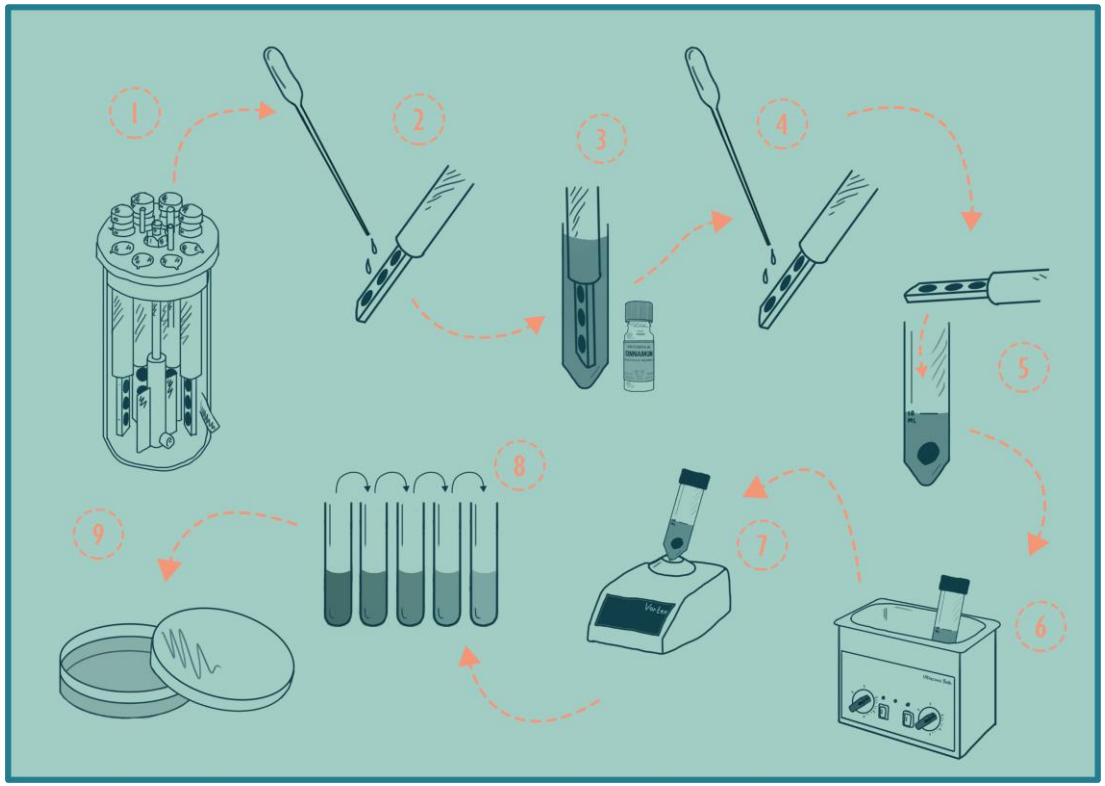


Figure 4.1 – Liquid Application Biofilm Treatment Method

Schematic of method for direct treatment of biofilms grown on coupons in the CBR. Biofilms are grown in the CBR (1), coupon holder rod is removed and rinsed in PBS (2). Biofilms are then exposed to cinnamon bark EO (3), followed by a PBS rinse (4). Single coupon is released from rod and placed in TSB (5), sonicated for 10 min (6) and vortexed for 30 s (7). Sample is serially diluted (8) and plated on agar for enumeration (9). Image created by author.

4.3 Results

4.3.1 CV biofilm assay

Results of the CV biofilm assay are demonstrated in Table 4.1. All bacteria tested were capable of forming biofilms, and categorised as either moderate or strong biofilm formers, with the exception of *A. baumannii* ATCC 17978 which was classified as weak.

Table 4.1 – Biofilm Adherence Testing

Classification of biofilm adherence of various bacterial strains following the crystal violet biofilm assay. *N=3*

Organism	**Absorbance (OD_{595})**	**Adherence**
Negative control	0.082	N/A
Escherichia coli ATCC 23848 (B strain)	0.244	Moderate
Escherichia coli NCTC 9001	0.108	Weak
Staphylococcus aureus ATCC 6358	0.887	Strong
Staphylococcus aureus NCTC 12981	3.903	Strong
Hospital Acquired MRSA isolate	1.460	Strong
MSSA NCTC 13297	1.664	Strong
Acinetobacter baumannii NCTC 12156	0.617	Strong
Acinetobacter baumannii ATCC 17978	0.629	Strong
*Pseudomonas aeruginosa (*PAO1)	3.920	Strong
Pseudomonas aeruginosa NCTC 8505	2.641	Strong

4.3.2 MBIC and MBEC

Results from MBIC testing and a summary of results from MBIC and MBEC testing can be seen in Table 4.2 and Table 4.3, respectively. *P. aeruginosa* (PAO1) biofilms grown in the presence of EO concentrations greater than or equal to 0.12% (v/v) were categorised as non-adherent, biofilms grown in the presence of 0.06% (v/v) EO were moderately adherent, and biofilms grown in the presence of EO concentration less than or equal to 0.03% (v/v) were strongly adherent. Thus, the minimum concentration of cinnamon bark EO which effectively inhibited the growth of *P. aeruginosa* biofilm was determined to be 0.12% (v/v). Preformed biofilms treated with cinnamon EO were effectively removed when treated with EO concentrations greater than or equal to 1% (v/v). However, when treated biofilms were provided with fresh medium, bacterial viability was evident after 6 h of further incubation in biofilms treated with 1% (v/v) EO. No regrowth was seen in biofilms that were treated with 2% (v/v) EO. Therefore, the minimum concentration of cinnamon bark EO which effectively eradicated preformed *P. aeruginosa* biofilms, with no regrowth after further incubation in fresh medium, is 2% (v/v).

Table 4.2 – Minimum Biofilm Inhibition Concentration Testing

Classification of biofilm adherence of *P. aeruginosa* PAO1 in the presence of cinnamon bark essential oil. *N=3*

Concentration of cinnamon bark EO	Absorbance (OD_{595})*	Adherence
Negative control	0.054	N/A
4%	0.000	Non-adherent
2%	0.014	Non-adherent
1%	0.038	Non-adherent
0.5%	0.026	Non-adherent
0.25%	0.022	Non-adherent
0.125%	0.034	Non-adherent
0.06%	0.195	Moderate
0.03%	0.915	Strong
0%	2.219	Strong

* Values corrected to subtract absorbance values of oil dilutions

Table 4.3 – Biofilm Inhibition and Eradication Testing

Minimum biofilm inhibition concentration (MBIC) and minimum biofilm eradication concentration (MBEC) values for cinnamon bark EO against *P. aeruginosa* PAO1

	Cinnamon bark EO concentration (v/v)
MBIC	0.12%
MBEC	2%

4.3.3 Direct treatment of biofilms on polycarbonate (PC)

Results of treating *P. aeruginosa* biofilms grown on PC coupons with cinnamon bark EO in liquid state is shown in Figure 4.2, Figure **4.3** and Figure **4.4**. After 24 h of contact time, treatment with 0.02% (v/v) had no significant effect on biofilms ($P>0.05$), however, biofilms treated with either 0.2% (v/v) or 2% (v/v) were significantly reduced after 24 h of treatment ($P<0.0001$). Treatment with 0.2% (v/v) resulted in a 5.22 log reduction of viable bacteria whereas 2% (v/v) resulted in 8 log reduction and complete eradication of biofilm (100% reduction). Further testing for 0.2% and 2% (v/v) treatment included 10, 30 and 60 min, and 6 and 12 h contact times (Figure 4.3 and Figure 4.4). After 10, 30 and 60 min exposure to 0.2% (v/v), less than 2 log reduction in CFU mL^{-1} was observed in biofilms grown on PC coupons. After 6 h and 12 h, 3.39 and 3.84 log reductions were observed, respectively. Exposure to 2% (v/v) again produced 100% reduction after only 10 min contact time and this was maintained across all further contact times (Figure 4.4).

Further testing for 2% (v/v) treatment included a shorter 5 min contact time and a 3 log reduction was observed (Figure 4.4).

4.3.4 Direct treatment of biofilms on stainless steel (SS)

Results of *P. aeruginosa* biofilms grown on SS coupons treated with cinnamon bark EO in liquid state are shown in Figure 4.2 and Figure 4.5. After 24 h of contact time, treatment with 0.02% (v/v) had no significant effect on biofilms ($P>0.05$). Biofilms treated with 0.2% (v/v) for 24 h were significantly reduced by 3.9 log ($P \leq 0.0001$), and biofilms treated with 2% (v/v) for 24 h were significantly reduced by 4.19 log ($P<0.001$). Further testing for 2% (v/v) treatment included 10, 30 and 60 min, and 6 and 12 h contact times. A 3.28, 3.34 and 3.34 log reduction was observed in biofilms treated with 0.2% (v/v) for 10, 30 and 60 min, respectively, and 4.45 and 4.46 log reductions were observed, after 6 h and 12 h, respectively. Log reductions after 10 min, 60 min and 6 h were significantly different from the control ($P<0.0001$), as were log reductions after 30 min and 12 h ($P<0.001$).

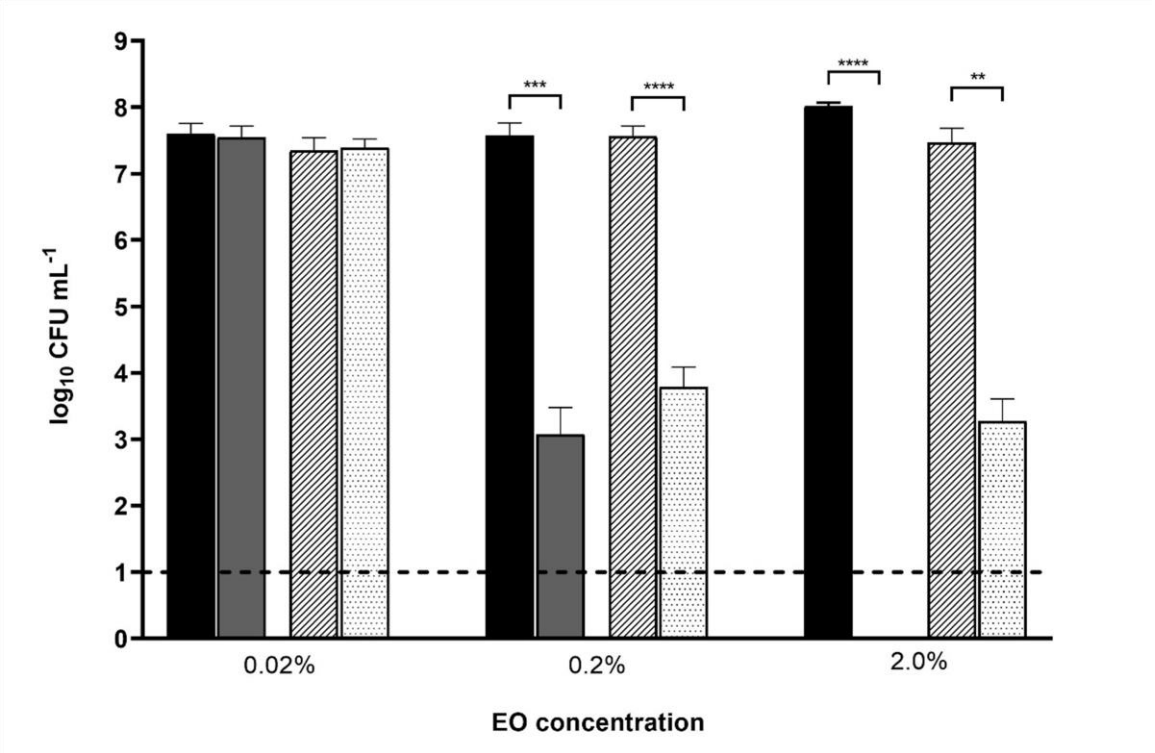


Figure 4.2 –Biofilm Treatment (24 h) with Cinnamon Bark EO

Log CFU mL^{-1} from disaggregated *P. aeruginosa* PAO1 biofilms grown on polycarbonate (PC) and stainless steel (SS) coupons for 48 h, and then either untreated or treated with cinnamon bark essential oil for 24 h at concentrations of 0.02%, 0.2% and 2% v/v. ■ untreated PC biofilms; ■ treated PC biofilms; ▨ untreated SS biofilms; ⬚ treated SS biofilms. N=3; bars show standard error; asterisks show statistical significance; dotted line indicating limit of detection = 10 CFU mL^{-1}.

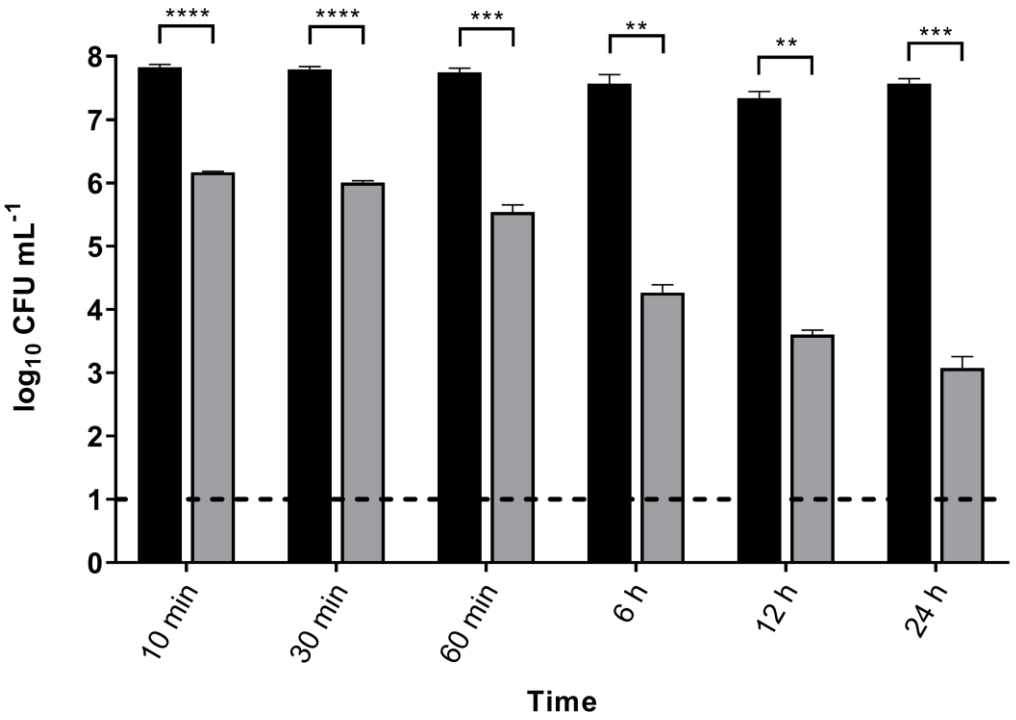


Figure 4.3 – Treatment of Biofilms on PC Coupons with Cinnamon Bark EO (0.2% v/v)

Log CFU mL^{-1} from disaggregated *P. aeruginosa* PAO1 biofilms grown on polycarbonate (PC) coupons for 48 h, and then either untreated or treated with cinnamon bark essential oil for 10 min to 24 h at a concentration of 0.2% v/v. ■ untreated PC biofilms; ▨ treated PC biofilms. N=3; bars show standard error; asterisks show statistical significance; dotted line indicating limit of detection = 10 CFU mL^{-1}.

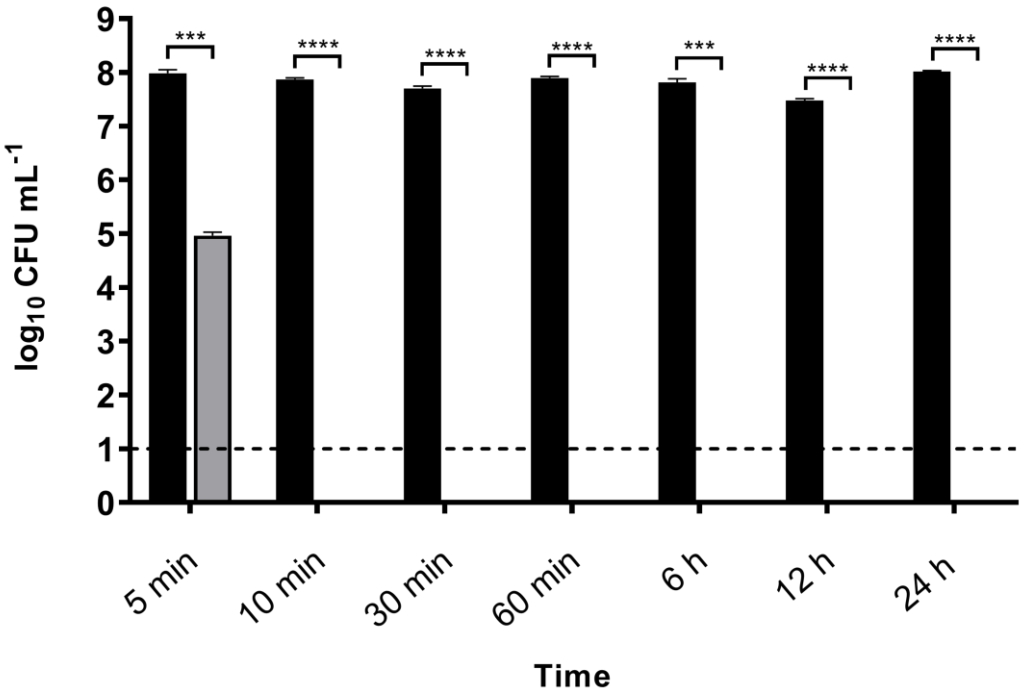


Figure 4.4 – Treatment of Biofilms on PC Coupons with Cinnamon Bark EO (2% v/v)

Log CFU mL^{-1} from disaggregated *P. aeruginosa* PAO1 biofilms grown on polycarbonate (PC) coupons for 48 h, and then either untreated or treated with cinnamon bark essential oil for 10 min to 24 h at a concentration of 2% v/v. ■ untreated PC biofilms; ■ treated PC biofilms. N=3; bars show standard error; asterisks show statistical significance; dotted line indicating limit of detection = 10 CFU mL^{-1}.

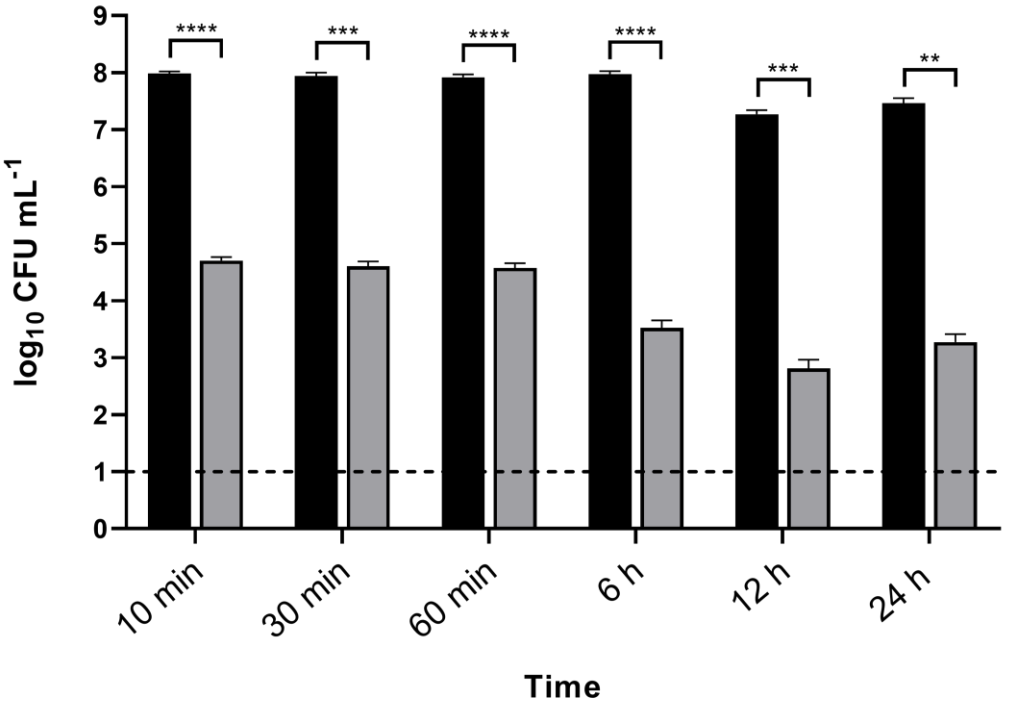


Figure 4.5 – Treatment of Biofilms on SS Coupons with Cinnamon Bark EO (2% v/v)

Log CFU mL^{-1} from disaggregated *P. aeruginosa* PAO1 biofilms grown on stainless steel (SS) coupons for 48 h, and then either untreated or treated with cinnamon bark essential oil for 10 min to 24 h at a concentration of 2% v/v. ■ untreated SS biofilms; ■ treated SS biofilms. N=3; bars show standard error; asterisks show statistical significance; dotted line indicating limit of detection = 10 CFU mL^{-1}.

4.4 Discussion

Due to the importance of the role of biofilms in AMR, this chapter aimed to investigate the biofilm forming capabilities of several species of bacteria and assess the anti-biofilm activity of cinnamon bark EO. Investigations began with an assessment of adherence using the crystal violet (CV) biofilm assay. The CV biofilm assay can be considered to be a semi-quantitative method and useful as a comparative tool to classify biofilm production (Di Domenico *et al.* 2016). Although the previous chapter focused on the effects of cinnamon bark EO against *P. aeruginosa* PAO1, the other species and/or strains of bacteria previously used were also included in the CV biofilm assay in this chapter. This was because *P. aeruginosa* is a known biofilm former, therefore the CV biofilm assay employed in this study not only confirmed this for the PAO1 strain, but also compared it to the other strain of *P. aeruginosa* and other species of bacteria used earlier in this study. Although all of the bacteria assessed in this chapter were capable of forming biofilms of varying strength, the knowledge that *P. aeruginosa* PAO1 formed strongly-adherent biofilms, coupled with previous findings from chapter 4 and 5 showing *Pseudomonas* to be the least tolerant to essential oils, justified the continued testing to assess anti-biofilm properties of cinnamon EO against only *Pseudomonas* biofilms. Use of the CV biofilm assay has its strengths and weaknesses for assessing biofilms and their treatment. Some of the main strengths of the CV biofilm assay are its simplicity, low cost, and ability to rapidly assess multiple samples at the same time (Wilson *et al.* 2017). The main disadvantage is that CV is a positively charged dye which will stain the surface of any negatively charged molecules, including the polysaccharide extracellular matrix of bacteria (Petrachi *et al.*

2017). Thus the assay indicates the amount of biomass that is left behind regardless of whether cells are living or dead (Pantanella *et al.* 2013). Nevertheless, the initial use of multi-well plates for biofilm growth provided a reliable high-throughput assay method for testing the biofilm forming capabilities of the bacteria used in this study.

The microtitre plate method of biofilm growth is a frequently used closed system model of growth (Macià *et al.* 2014), which can be easily adapted for anti-biofilm testing. However, it also comes with advantages and disadvantages similar to those of the CV biofilm assay. Using the microtitre plate biofilms for antimicrobial testing gives the user the ability to test multiple species, several treatments and/or growth conditions, changes in temperature and humidity, or all of the above, at the same time (Coenye and Nelis 2010). Unfortunately, its closed system with no inward or outward flow means that uncontrolled changes will happen to the environment within the wells of the plate, including nutrient depletion, oxygen limitation, accumulation of metabolites or other molecules, and interference from planktonic cells (Merritt *et al.* 2011, Lüdecke *et al.* 2014, Macià *et al.* 2014, Azeredo *et al.* 2017). Despite these downfalls, this method was used first in this chapter to gain a preliminary understanding of the concentrations that might be required for later testing against biofilms.

The MBIC of cinnamon bark EO was determined as 0.12% (v/v), which is the same as the minimum inhibitory concentration of this EO against planktonic cells of this bacterium (chapter 3). This is similar to the data of Firmino *et al.* (2018), who found that cinnamon oil as low as 0.12 mg mL^{-1}, approximately 0.012% (v/v), reduced biofilm growth of P. aeruginosa after 24 h of culture

when assessing biomass, and 1.92 mg mL^{-1}, approximately 0.192% (v/v), prevented biofilm growth entirely.

With regards to MBEC methods in this study, the use of subculturing allowed an estimation of the viability of the bacteria following exposure to the EO. Methods used in this chapter employed a further 6 h incubation in fresh medium, however the duration of re-incubation could have been improved to better reflect real-time generation of *Pseudomonas* biofilms. Pericolini *et al.* (2018) report that whilst the generation time for *P. aeruginosa* PAO1 is less than 30 min, the maximum biofilm biomass is reached after 24 h incubation. Therefore, to ensure that a biofilm has been truly eradicated and unable to re-establish as a biofilm, a longer re-incubation time would be required following treatment. However, further incubation for 6 h, as was used here, can be used to comment on whether or not viable bacteria remain. These bacteria then have the potential to re-establish a biofilm.

Results from this chapter revealed that whilst 1% (v/v) EO was able to remove preformed *P. aeruginosa* biofilms, the remaining bacteria were viable and able to recover after further incubation with fresh media. Thus, the MBIC of cinnamon bark EO was determined as 2% (v/v) when used to treat *P. aeruginosa* PAO1 biofilms.

CFU counts are a simple and effective indicator of viability, more robust and thorough testing could be performed to give a definitive indication of viability. It has been reported that methods for assessing viability should take into consideration the method of antimicrobial testing, and ideally a combination of these methods could be utilised (Cai *et al.* 2014). These include indirect methods requiring further culturing or reactions, such as the CFU

determination or detection of metabolic activity using stains and indicators, or direct methods which use molecular probes such as the *Bac*Light™ Live/Dead stain kit (Cai *et al.* 2014). Whilst using these methods might be preferable, they were deemed unnecessary for the present study. This is because, although valuable, the use of microtitre plates for biofilm growth is not relatable to naturally occurring biofilms due to many of the disadvantages covered earlier. To address this, the anti-biofilm activity of cinnamon EO was investigated further using the CDC biofilm reactor (CBR).

The CBR was developed by Donlan *et al.* (2002) to provide a biofilm model that operates as a continuous-flow stirred tank reactor. Holders within the reactor are capable of accommodating 24 coupons, made of any chosen material, on which bacteria will attach and form biofilms. The reactor sits upon a stir plate, which can provide heat if required, and generates a shear force across the surface of the coupons by way of a magnetic baffle. Rigorous statistical assessments of the reactor ruggedness and repeatability have been examined by Goeres *et al.* (Goeres *et al.* 2005) who found that the CBR is a reliable model for biofilm growth and a useful tool in answering diverse research questions regarding biofilms. In fact, the recently produced American standards for biofilm disinfection uses the CBR as the primary growth model (Environmental Protection Agency 2017). Buckingham-Meyer *et al.* (2007) highlight the importance of choosing a biofilm model that will closely replicate the natural environment that the test bacteria typically are associated with, where several factors including shear force will create diverse and dynamic growth conditions.

P. aeruginosa is ubiquitous in the environment and commonly found in water, soil or associated with animals (Fazeli *et al.* 2012). This organism has been synonymous with hospital acquired infections for over 50 years and evidence has shown that hospital water plumbing networks are implicated in these endemic outbreaks (Quick *et al.* 2014). *P. aeruginosa* are regularly found in hospital water samples (Asghari *et al.* 2013) and generally harboured in and on water-related sites such as taps, sinks, showers, ice makers, mop heads and buckets, hydrotherapy pools and bath toys (Kerr and Snelling 2009, Aspelund *et al.* 2016, Bédard *et al.* 2016, Lalancette *et al.* 2017). This is evidence that *Pseudomonas* biofilms are likely to thrive in conditions where they are subjected to shear forces and continuous flow, and thus the CBR is ideal for use as a growth model to study this organism. It has been shown that biofilms grown using the CBR are not as easily removed compared to other models (Buckingham-Meyer *et al.* 2007) but that CBR biofilms are better models for removal with good relevance to industrial or clinical settings (Shelobolina *et al.* 2018).

Testing using the CBR provided evidence of the inefficiency of cinnamon bark EO to reduce PAO1 biofilms at very low concentrations (i.e. 0.02% v/v), when in liquid state, and showed cinnamon EO was unable to significantly reduce biofilms grown on either PC or SS surfaces at this concentration. This is a reasonable finding considering results from the MBEC testing performed earlier. However, this study demonstrated that low concentrations of cinnamon EO (i.e. 0.2% v/v) were capable of producing at least 3 log reduction in biofilms grown on either PC or SS after 24 h of contact time.

This reduction in biofilms is not only significant statistically ($P<0.001$) but also significant when considering cinnamon EO as a potential disinfectant *in situ.* Disinfection protocols are deployed routinely in the prevention and reduction of infection spread (Muniesa *et al.* 2019) and should conform to standards such as the British Standards European Norm (BS EN). Currently, only the United States Environmental Protection Agency (EPA) have published standardized protocols for anti-biofilm efficacy testing, and there are no European standards in this area (Ledwoch *et al.* 2019). Therefore, new disinfectants developed within the UK rely on the BS EN standards 1276 and 13697 for suspension and surface disinfection, though these are relative to planktonic state bacteria only. Consequently, if these standards are to be used as a benchmark for anti-biofilm efficacy then contact time should be 5 min and log reduction in CFU should exceed 5 (British Standards Institution 2009, 2015). In 2017 the EPA became the first agency to offer methods and guidance in combatting biofilms growing on hard non-porous surfaces, and recommended that a successful product should reduce biofilms by at least 6 log CFU, with a minimum starting bacterial load of 8 log CFU, and a contact time not exceeding 10 min (Environmental Protection Agency 2017). Whilst investigating the anti-biofilm effects of cinnamon bark EO within this chapter, both of these standards were considered when evaluating successes and/or failures of cinnamon bark EO.

Further testing to investigate 2% (v/v) EO proved the oil to be even more effective and showed that cinnamon EO was able to cause significant reduction of biofilms within 5 and 10 min when grown on PC and SS, respectively. When grown on PC coupons, biofilms exposed to 2% (v/v) EO

were reduced by 3.02 log CFU mL^{-1} after 5 min of contact time ($P<0.001$). Following exposure to the EO for 10 min or more, biofilms on PC coupons were reduced completely and no viable bacteria were seen in further cultivation steps ($P<0.001$). In contrast to this, biofilms grown on SS coupons remained viable after all durations of exposure to 2% (v/v) EO. After 10 min contact time, a 3.28 log reduction in CFU mL^{-1} ($P<0.001$) was observed. Despite further reduction as duration of treatment increased, after 24 h of contact time ~44% of bacteria remained viable on the coupon, compared to that of 0% viable bacteria on PC coupon biofilms tested in comparable conditions.

In the previous chapter it was noted that the toxicity of cinnamon bark oil would need to be taken into consideration when evaluating its uses. In this chapter, cinnamon oil at 2% was highly effective, which is lower than the described concentration that could cause skin irritation (Bickers *et al.* 2005), thus providing promise in its use which could potentially lead to contact with human skin.

The present study grew biofilms on two different surfaces, polycarbonate plastic (PC) and 316L stainless steel (SS). Both of these materials are commonly used surface materials, especially in hospital environments (Schmidt *et al.* 2012). Saka *et al.* (2017) report that microbial pathogens can survive for extended periods of time on both SS and polymeric materials. The present study has indicated that cinnamon EO appeared to be more effective at reducing biofilms when they were grown on PC as opposed to SS coupons. This may be explained by a difference in surface attachment between biofilms on PC and SS. Results from the study by Abdallah *et al.* (2014), indicate that

when *P. aeruginosa* was grown on SS, the adhesion rate was double that of biofilms grown on PC. This is possibly connected to surface smoothness, as a rougher surface could play a role in adhesion by offering protection against shear forces (Barnes *et al.* 1999). Baker (1984) also found that when investigating bacterial attachment in rivers, roughened substrates were colonised significantly faster than the smoother surfaces. Baker speculates if this is due to rougher surfaces providing protection, but also suggests that roughened surfaces would have an increased surface area, providing a larger area for colonization. A study by Holah and Thorpe (1990) used scanning electron microscopy to assess the surface roughness of both PC and SS surfaces, and found untampered PC to be the smoothest. These reports corroborate findings from this study and emphasize the importance of surface of growth when testing antimicrobial efficacy against biofilms.

In conclusion, this study aimed to address the question of how effective cinnamon bark EO is against *P. aeruginosa* biofilms. Preliminary biofilm models utilised microtitre plates for high-throughput and multiple treatment environments. This found the MBIC to be a low concentration of 0.125% (v/v) and showed an MBEC of 2% (v/v). Continuing from these tests, the CBR was employed to produce robust mature biofilms which more closely reflected those that are naturally occurring. The anti-biofilm effects of cinnamon bark EO were tested against biofilms grown on either PC plastic or SS surfaces, mimicking surfaces commonly found in both industrial and healthcare settings. Cinnamon bark EO at 2% (v/v) proved to be the most effective, especially against biofilms grown on PC coupons, and successfully reduced these biofilms by 100% after 10 min of contact time. A 5 min contact time of 2% (v/v)

EO against biofilms on PC also reduced biofilms by 3 log CFU mL^{-1}. It was shown that cinnamon bark EO was more effective against biofilms grown on PC versus SS and this could be explained by the microscopic differences between these two surfaces and how bacteria may interact with them. If US standards for anti-biofilm activity are used to evaluate the findings from this study, then cinnamon bark EO is an eligible product for biofilm disinfection, producing a greater than 6 log reduction within 10 min of contact time. Nonetheless, it is evident that European standards need to be reviewed and updated to include the growth of bacteria as biofilms that are ubiquitous in the environment.

CHAPTER 5

5 VAPOUR CHAMBER DEVELOPMENT AND TREATMENT OF *PSEUDOMONAS AERUGINOSA* BIOFILMS WITH CINNAMON BARK ESSENTIAL OIL VAPOUR.

5.1 Introduction

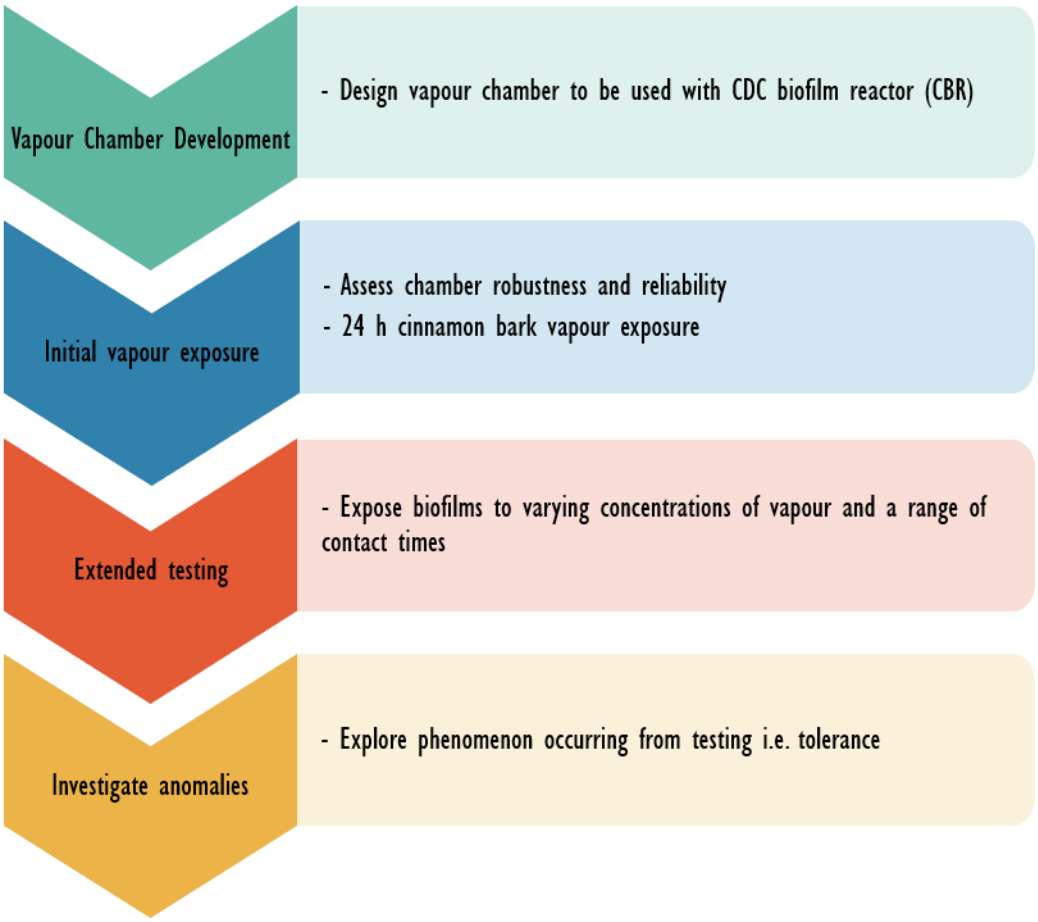
Essential oils (EOs) are well known for their volatility and have long been utilised in their vapour state. The use of EO vapour as an antimicrobial has been well documented in the literature (Doran *et al.* 2009, Fisher and Phillips 2009, Nedorostova *et al.* 2009, Nostro *et al.* 2009, Tyagi and Malik 2010a, Velázquez-Nuñez *et al.* 2013) and several studies also include cinnamon EO vapour (Inouye *et al.* 2001, López *et al.* 2007, Goñi *et al.* 2009, Seo *et al.* 2015, Ghabraie *et al.* 2016, Feyaerts *et al.* 2018, Lee *et al.* 2018). Methods that are usually employed in these studies include the use of the disc-volatilisation method which often comes under many other pseudonyms. Regardless of the name given, this method usually involves the use of a petri-dish containing organisms growing on solid agar, which is then inverted and the EO is placed in the lid to assess antimicrobial vapour effects (Doran *et al.* 2009), similar to disc diffusion assays seen previously in this thesis (Chapter 2). The space between the agar surface and the lid can then be calculated and thus vapour can be expressed per litre of air, though this is not an accurate representation of concentration (Kloucek *et al.* 2012). Others have gone further and created environments for vapour testing using sealed boxes, usually plastic or glass, and usually a much larger volume. For example, Fisher and Phillips (2009) used a 600 L capacity sealed box to test a blend of citrus EOs against bacteria growing in broth cultures. Inouye *et al* (2001) utilised 1.3 L capacity foil lined

plastic boxes to test bacteria growing on solid agar plates. Doran *et al* (2009) studied the effects of a combination of lemongrass and geranium EO in a similar manner using 64 L capacity boxes and, further to this, tested the effects of this EO mix on airborne microbes in office spaces by utilising an ST Pro™ machine to disperse EO throughout a 25 m^2 room. However, whilst all these methods are well placed in testing the potential of EO vapours as antimicrobials, all these studies fail to assess the EO vapour effect against biofilms. Studies that look at the antibiofilm properties of EO vapour are extremely limited.

Nostro *et al* (2009) exposed biofilms grown in 35mm polystyrene plates to vapour of carvacrol, a common component of EOs. Laird *et al.* (2012) grew biofilms on 2 cm stainless steel discs and placed them in a 600 L capacity sealed box with a blend of orange and bergamot EO vapour. More recently, Benzaid *et al* (2019) treated *Candida albicans* biofilms, growing on collagen scaffolds, with peppermint (*Mentha* × *piperita*) vapour. These three studies are the limit to EO vapour versus biofilm work published to date, and these studies are limited by the use of static biofilm model systems. It is also noted that no study to date has investigated the effect of EO vapours against *P. aeruginosa* biofilms or of cinnamon bark EO vapour against biofilms. Therefore, the current chapter aimed to address the gaps within the literature and set out to develop a method of exposing *P. aeruginosa* biofilms, grown in a continuous flow biofilm model system, to cinnamon bark EO vapour.

5.1.1 Chapter progression

The following flow diagram depicts a summary of the progression of this chapter.



5.2 Methods

5.2.1 Growth of *P. aeruginosa* biofilms in the Centre for Disease Control biofilm reactor (CBR)

Standardised 48 h mature *P. aeruginosa* PAO1 biofilms were grown on polycarbonate coupons in the CBR as described in section 2.4.2.

5.2.2 Vapour chamber development and biofilm treatment

A novel custom-made vapour chamber (Figure 5.1) was developed for treating biofilms and adapted for use with a CBR. Due to the configuration of the CBR, it was necessary to design a chamber that would accommodate the coupons, making it possible to treat them with vapour, but also minimising their disruption. This would most likely be achieved by avoiding the removal of the coupons from their rods. A feature of the CBR is easily removed rods for sampling, thus an airtight plastic receptacle with a shape that replicated the CBR could be used and adapted so as to accommodate the rods. This would facilitate the easy removal of rods from the CBR and replacement in this modified chamber, followed by subsequent treatment using vapours. It was anticipated that further analysis may be needed on the vapours within the chamber, thus a method of accessing the interior environment without disturbing the vapour equilibrium or the biofilms was needed. The final chamber design consisted of a 1.3 L volume (1300cm^3) plastic container, measuring 11 x 27 cm, and featured a removable airtight lid. The container was orientated so that this lid subsequently became the base, and the top of the container was adapted to include four ports in which rods from the reactor fit snuggly, with a fifth smaller central hole in which a silicon septum was fitted. Vapour chambers were wrapped in foil, sterilised by autoclaving and allowed to dry before use. A schematic of the method for vapour treatment of mature biofilms is demonstrated in Figure 5.2. Briefly, coupon holder rods were removed from the reactor aseptically and rinsed with sterile PBS. After rinsing, rods were carefully inserted into the upper ports of the sterile vapour chamber. In circumstances where all four ports were not filled with rods containing

coupons, a sterile blank coupon holder was used to fill the space, to ensure a closed unit for vapour treatment.

For treatment using cinnamon bark essential oil, a sterile 47 mm filter paper disc (Whatman, UK) was placed in a sterile 50 mm glass petri dish and placed in the bottom of the vapour chamber. The filter paper was then saturated with 260 µL of cinnamon EO, representing a concentration of 0.2 µL cm^{-3} and the vapour chamber was immediately sealed. A separate negative control vapour chamber had a petri dish with only filter paper present. The chambers were incubated at 37°C for 24 hours, after which, rods were aseptically removed, rinsed in sterile PBS, and each coupon released into a separate tube containing 10 mL of TSB. Coupons were then sonicated for 10 min and vortexed for 30 seconds. The disaggregated biofilms were then serially diluted and plated onto MHA and incubated at 37°C for 24 h. During initial development of the vapour chamber, the position of the coupon within the chamber was assessed for influence on biofilm CFU. The position of coupon was noted, i.e. top, middle or bottom, and three coupons were enumerated for each position for test condition and control and the experiment was carried out in triplicate. Initial testing to assess coupon placement was carried out on biofilms grown on polycarbonate coupons and using EO concentration of 0.2 µL cm^{-3}.

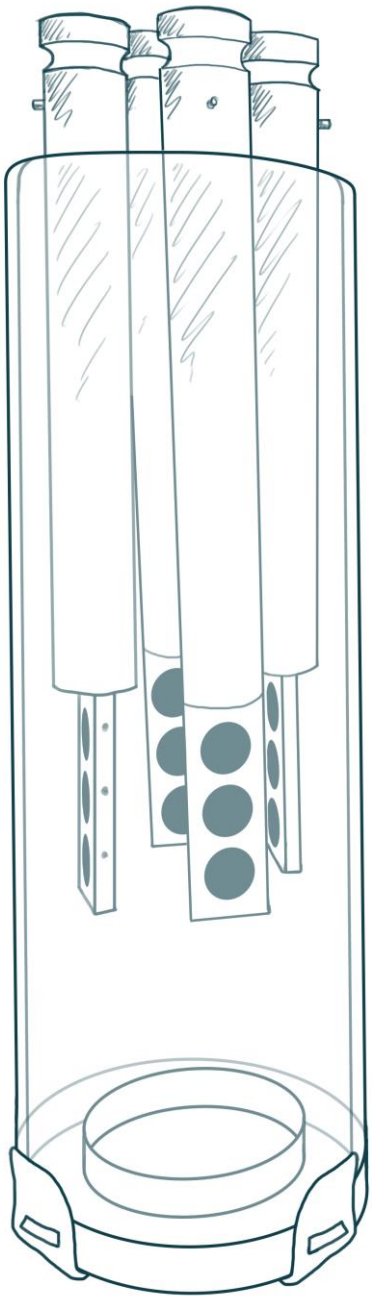


Figure 5.1 – Vapour Chamber

Diagram of 1.3 L capacity vapour chamber used for vapour treatment of biofilms grown in the CDC biofilm reactor. Image created by author.

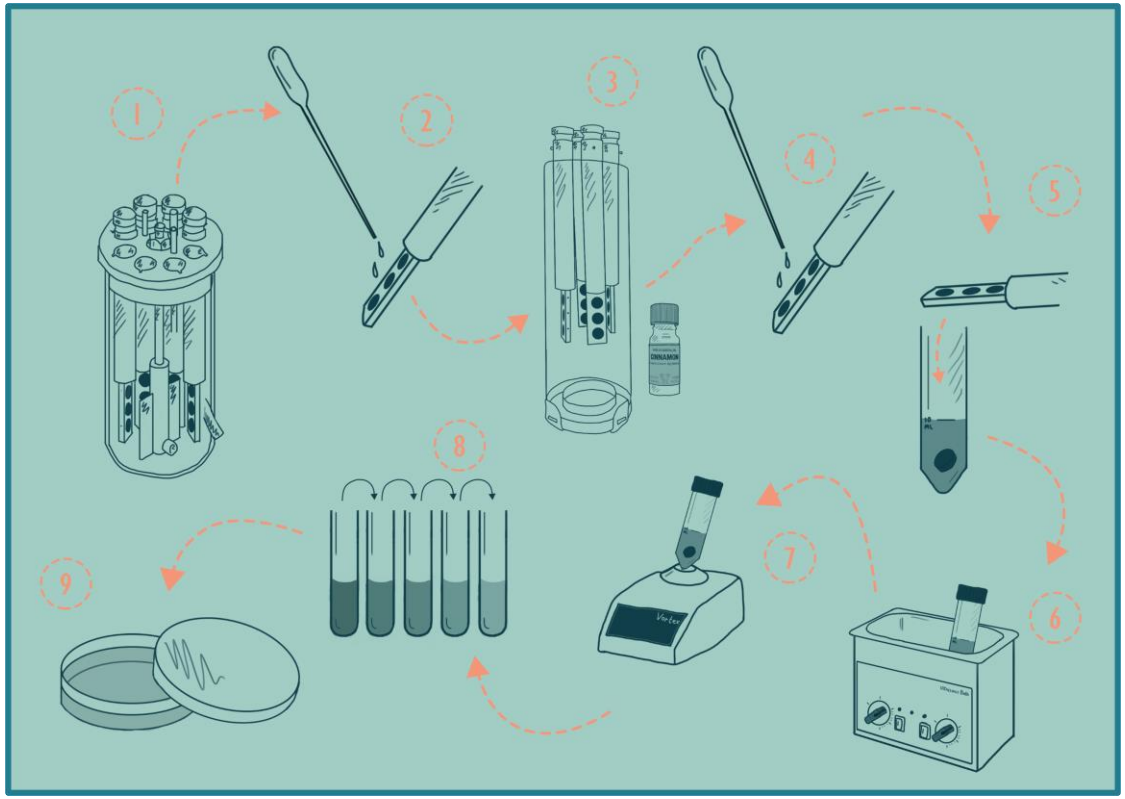


Figure 5.2 - Liquid Application Biofilm Treatment Method

Schematic of method for direct treatment of biofilms grown on coupons in the CBR. Biofilms are grown in the CBR (1), coupon holder rod is removed and rinsed in PBS (2). Biofilms are then exposed to cinnamon bark EO vapour (3), followed by a PBS rinse (4). Single coupon is released from rod and placed in TSB (5), sonicated for 10 min (6) and vortexed for 30 s (7). Sample is serially diluted (8) and plated on agar for enumeration (9). Image created by author.

5.2.3 Further vapour treatment

Following confirmation that coupon position within the vapour chamber did not influence CFU of biofilms, further vapour treatments were carried out on biofilms grown on either polycarbonate (PC) or stainless steel (SS) coupons using similar methods to those described in section 6.2.2, with the following adaptions. The filter paper was placed at the bottom of the chamber and saturated with either 260 µL or 2.6 mL of cinnamon EO, representing concentrations of 0.2 µL cm^{-3} or 2 µL cm^{-3}, respectively. The untreated control chamber contained only filter paper. The chambers were incubated at 37°C and biofilms grown on PC coupons were exposed for 10, 30, 60 min and 6, 12 h and 24 hours, whilst biofilms grown on SS were exposed for 24 h only. After exposure to the EO vapour, rods were aseptically removed, rinsed in sterile PBS, and each coupon released into a separate tube containing 10 mL of TSB. Coupons were then sonicated for 10 min and vortexed for 30 seconds. The disaggregated biofilms were then serially diluted and plated onto MHA and incubated at 37°C for 24 h. Three coupons were enumerated for each condition and the experiment was carried out in triplicate.

5.2.4 Assessment of tolerance

Biofilms treated with 0.2 µL cm^{-3} cinnamon bark EO vapour showed complete reduction after 12 h of contact but then growth at 24 h time point. Following this observation, bacteria recovered from the 24 h time point were subcultured and named tolerance subculture 1 (TSC-1). Six h, 12 h and 24 h vapour treatment testing, as before (section 6.2.3), was carried out on TSC-1 to see if there was tolerance/resistance. Bacteria recovered from the 24 h time point

of TSC-1 testing, named tolerance subculture 2 (TSC-2), were also subcultured and tested in the same way. Three coupons were enumerated for each condition and the experiment was carried out in triplicate.

5.3 Results

5.3.1 Effect of coupon position in vapour chamber

Results following analysis of the effect of coupon position within the vapour chamber are shown in Figure 5.3. There was no significant difference observed between coupons placed at either top, middle or bottom position in either treated or untreated vapour chambers ($P>0.05$). Because of this observation, data for treated or untreated coupons were collated as can be seen in Figure 5.4. An overall significant log reduction of 5.60 ($P<0.0001$) was observed in CFU mL^{-1} after 24 h exposure to EO vapour at 0.2 µL cm^{-3}.

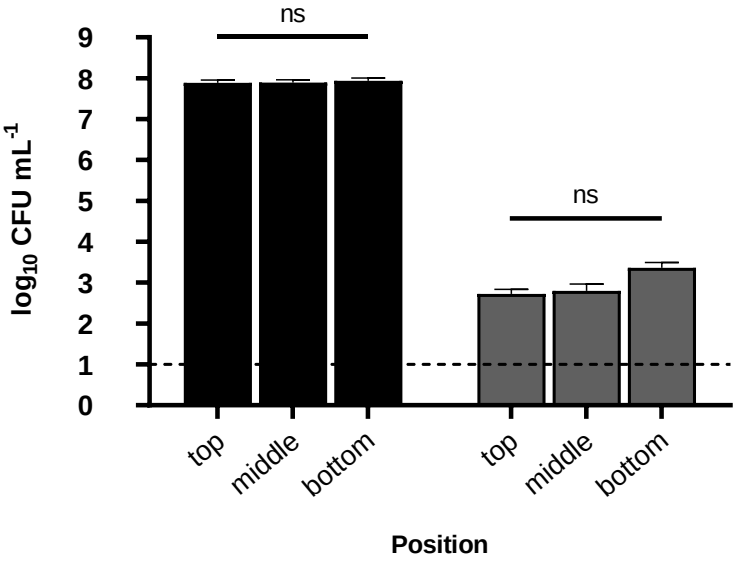


Figure 5.3 – Coupon Position Effect Assessment

Assessment of coupon position (top, middle, or bottom) within vapour chamber where *P. aeruginosa* PAO1 biofilms grown on polycarbonate coupons are untreated or treated with 0.2 µL cm^{-3} cinnamon bark essential oil vapour for 24 h. ■ untreated biofilms; ■ treated biofilms. Bars show standard error; *N*=3; ns = not significant; dotted line indicating limit of detection = 10 CFU mL^{-1}.

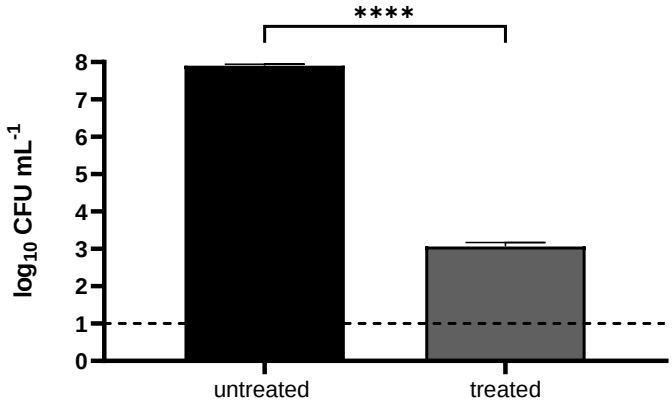


Figure 5.4 – Cinnamon Bark EO Vapour (0.2 µL cm^{-3}) Treated *P. aeruginosa* Biofilm (24 h)

P. aeruginosa PAO1 biofilms, grown on polycarbonate coupons, untreated or treated with 0.2 µL cm^{-3} cinnamon bark essential oil vapour for 24 h. ■ untreated biofilms; ■ treated biofilms. Bars show standard error; N=9; asterisks indicate significance; dotted line indicating limit of detection = 10 CFU mL^{-1}.

5.3.2 Further vapour treatment of *Pseudomonas* biofilms

Log reduction of biofilms grown on PC coupons following cinnamon bark vapour treatment at 0.2 µL cm^{-3} is shown in Figure 5.5. Log reduction after 10, 30 and 60 min of vapour treatment was minimal (<0.5 log). Overall log reduction at 6 h and 12 h was 7.50 and 7.76, respectively. Following 24 h of vapour exposure, log reduction was 5.60, suggesting lower reduction of biofilm CFU mL^{-1} compared to that seen at 6 h and 12 h.

Log reductions of biofilms grown on polycarbonate coupons following cinnamon bark vapour treatment at 2.0 µL cm^{-3} are shown in Figure 5.6. Log reductions after 10, 30 and 60 min of vapour treatment were minimal (<0.5 log). Log reduction at 6 h, 12 h and 24 h was significantly reduced by 3.84, 4.05 and 3.90, respectively ($P<0.0001$).

Log reductions of biofilms grown on stainless steel coupons following exposure to cinnamon bark EO vapour are shown in Figure 5.7 and Figure 5.8. Both concentrations produced a significant log reduction ($P<0.001$) in CFU mL^{-1}. For 0.2 and 2 µL cm^{-3}, cinnamon vapour produced 3.96 and 4.39 log reductions were produced, respectively.

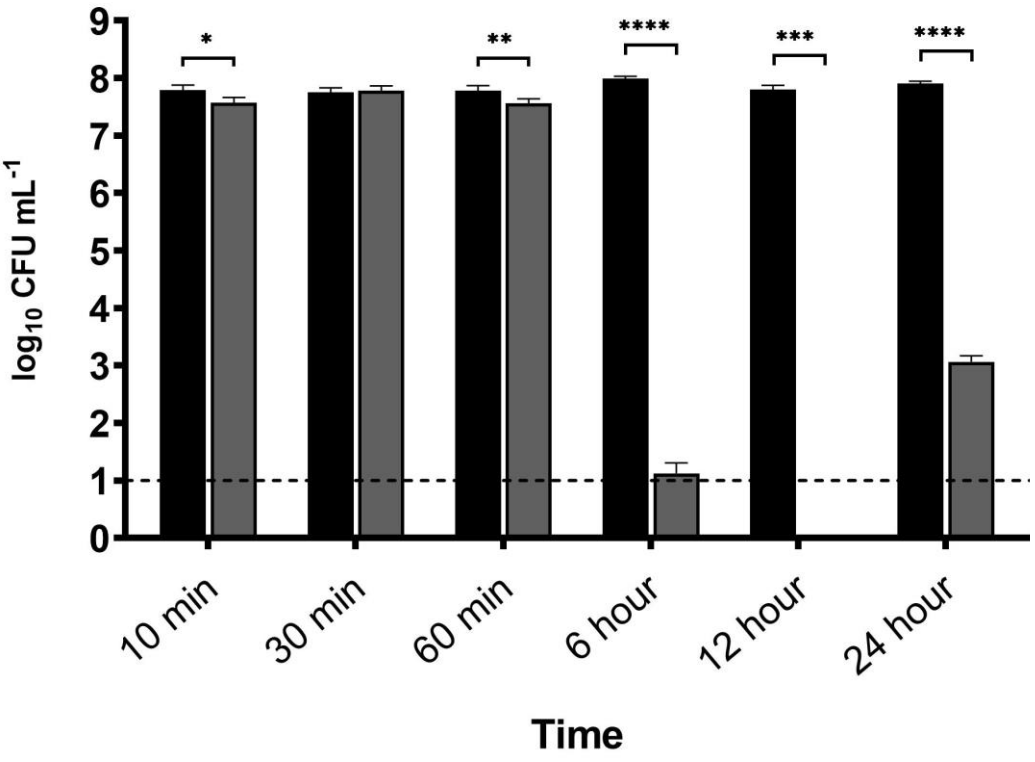


Figure 5.5 – Cinnamon Bark EO Vapour (0.2 µL cm^{-3}) Treated *P. aeruginosa* Biofilms Grown on PC.

P. aeruginosa PAO1 biofilms, grown on polycarbonate coupons, either untreated or treated with 0.2 µL cm^{-3} cinnamon bark essential oil vapour 10, 30 60 min and 6, 12 and 24 h. ■ untreated biofilms; ■ treated biofilms. Bars show standard error; *N*=3; asterisks indicate significance; dotted line indicating limit of detection = 10 CFU mL^{-1}.

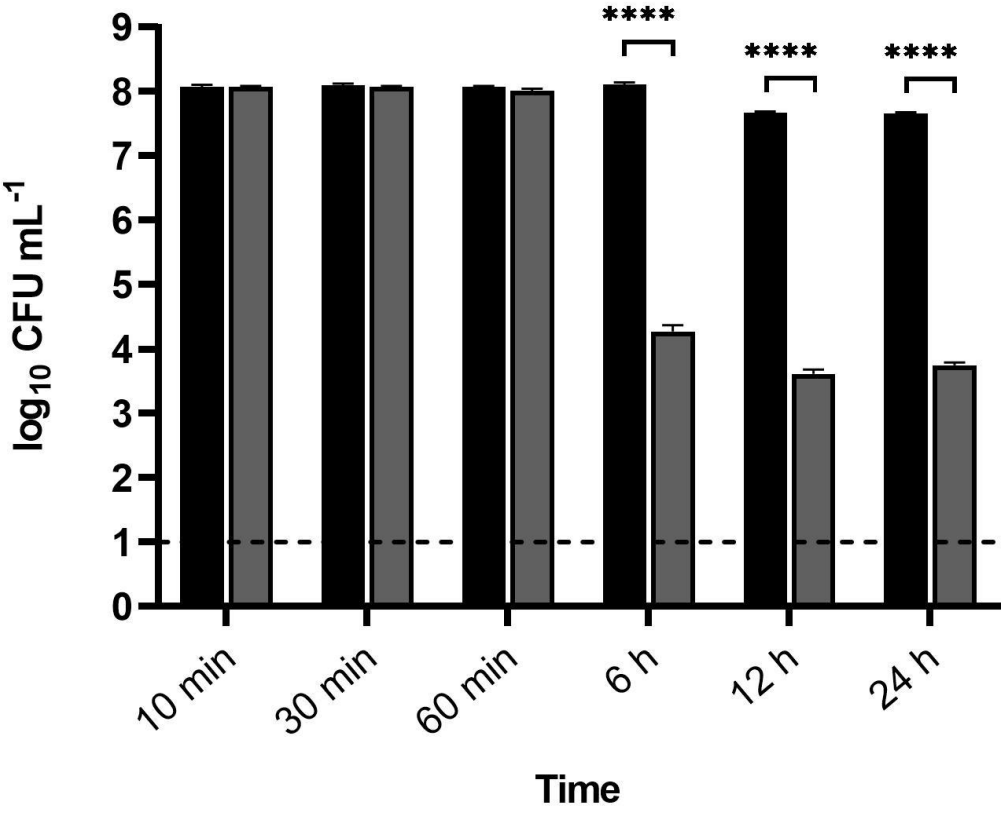


Figure 5.6 – Cinnamon Bark EO Vapour (2 µL cm^{-3}) Treated *P. aeruginosa* Biofilms Grown on PC.

P. aeruginosa PAO1 biofilms, grown on polycarbonate coupons, either untreated or treated with 2 µL cm^{-3} cinnamon bark essential oil vapour 10, 30 60 min and 6, 12 and 24 h. ■ untreated biofilms; ■ treated biofilms. Bars show standard error; *N*=3; asterisks indicate significance; dotted line indicating limit of detection = 10 CFU mL^{-1}.

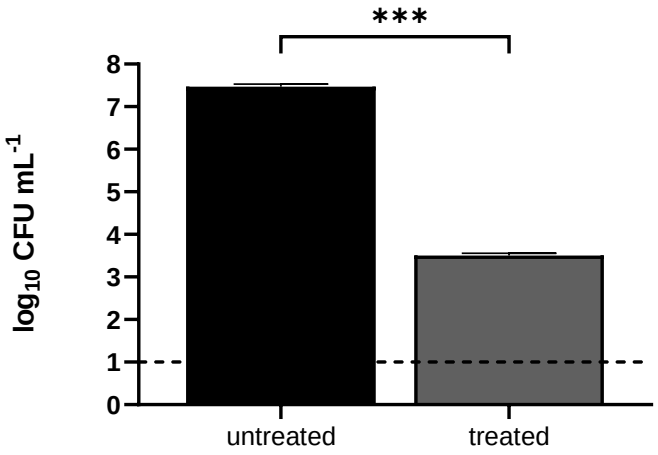


Figure 5.7 – Cinnamon Bark EO Vapour (0.2 µL cm^{-3}) Treated *P. aeruginosa* Biofilms Grown on SS.

P. aeruginosa PAO1 biofilms, grown on stainless steel coupons, untreated or treated with 0.2 µL cm^{-3} cinnamon bark essential oil vapour for 24 h. ■ untreated biofilms; ■ treated biofilms. Bars show standard error; N=3; asterisks indicate significance; dotted line indicating limit of detection = 10 CFU mL^{-1}.

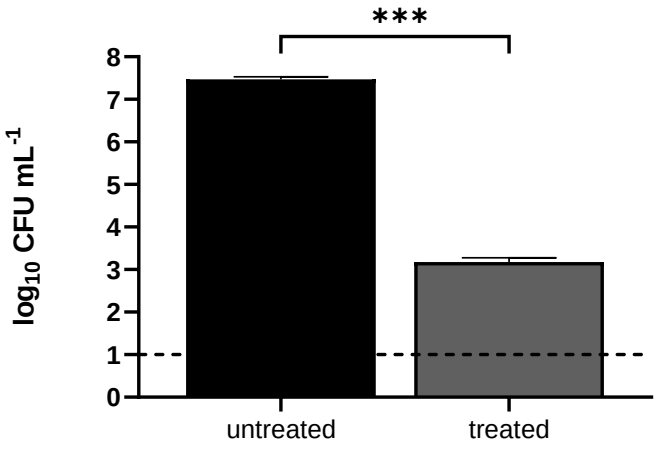


Figure 5.8 – Cinnamon Bark EO Vapour (2 µL cm^{-3}) Treated *P. aeruginosa* Biofilms Grown on SS.

P. aeruginosa PAO1 biofilms, grown on stainless steel coupons, untreated or treated with 2.0 µL cm^{-3} cinnamon bark essential oil vapour for 24 h. ■ untreated biofilms; ■ treated biofilms. Bars show standard error; N=3; asterisks indicate significance; dotted line indicating limit of detection = 10 CFU mL^{-1}.

5.3.3 Assessment of tolerance in *Pseudomonas* biofilms

Following observation of regrowth at the 24 h contact point, the tolerance subculture 1 (TSC-1) was subjected to further testing, with results of this shown in Figure 5.9. Log reduction of TSC-1 biofilms after 6, 12 and 24 h of exposure were 4.89, 5.55 and 5.17, respectively ($P<0.0001$). Tolerance subculture 2 (TSC-2) was also subjected to further testing, with results of this shown in Figure 5.10. Log reduction of TSC-2 biofilms after 6, 12 and 24 h of exposure were 5.03, 5.82 and 5.94, respectively ($P<0.0001$).

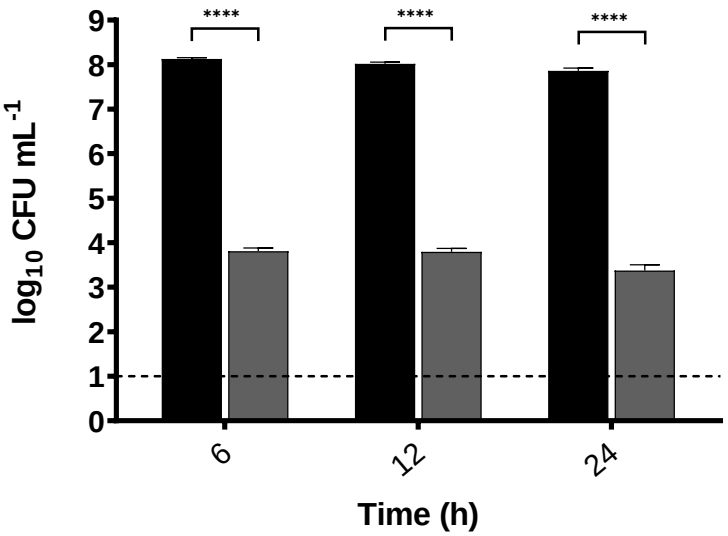


Figure 5.9 – Cinnamon Bark EO Vapour (0.2 µL cm^{-3}) Treated TSC-1 Biofilms Grown on PC.

Tolerance Subculture 1 (TSC-1) biofilms, grown on polycarbonate coupons, either untreated or treated with 0.2 µL cm^{-3} cinnamon bark EO for 6, 12 and 24 h. ■ untreated biofilm; ■ treated biofilm. Bars show standard error; *N*=3; asterisks indicate significance; dotted line indicating limit of detection = 10 CFU mL^{-1}.

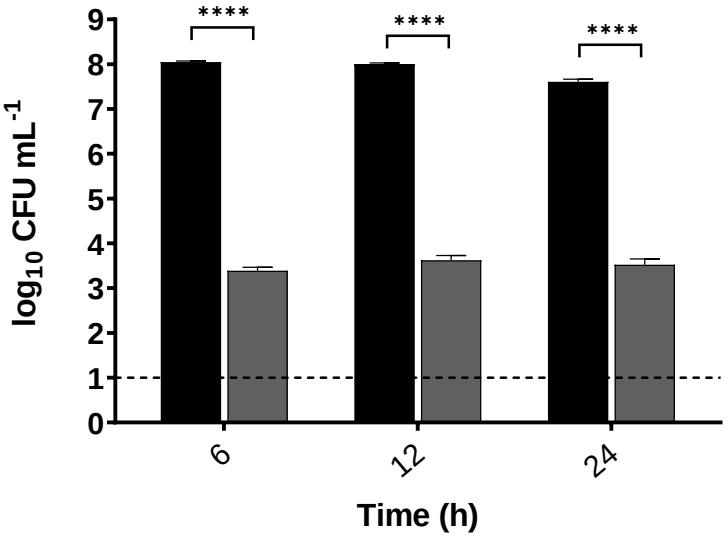


Figure 5.10 – Cinnamon Bark EO Vapour (0.2 µL cm^{-3}) Treated TSC-2 Biofilms Grown on PC.

Biofilms of Tolerance Subculture 2 (TSC-2) of *P. aeruginosa* PAO1, grown on polycarbonate coupons, either untreated or treated with 0.2 µL cm^{-3} cinnamon bark EO for 6, 12 and 24 h. ■ untreated biofilm; ■ treated biofilm. Bars show standard error; *N*=3; asterisks indicate significance; dotted line indicating limit of detection = 10 CFU mL^{-1}.

5.4 Discussion

The previous chapter investigated the effects of cinnamon bark EO in liquid phase and its efficacy against biofilms grown in the CDC biofilm reactor (CBR). This chapter aimed to investigate the anti-biofilm effects of the same EO in vapour phase. The first step to approach this was in the development of a suitable method of exposing biofilms grown using the CBR to cinnamon EO vapour, a biofilm model which has been advocated for biofilm growth previously (chapter 5). As previously discussed, other methods in the literature utilised sealed containers as vapour chambers to test the antimicrobial effects of EO vapours (Inouye *et al.* 2001, Doran *et al.* 2009, Fisher and Phillips 2009), thus, a unit was developed that would accommodate the coupon holder rods and enable treatment of biofilms growing on the coupons *in situ.* As this was a novel invention, steps were carried out to ensure that the model was robust and reliable in its purpose. With the understanding that as EO liquid is heated, the volatile components in their gaseous state will rise above the liquid in a general upwards direction (Clark 2009), consideration had to be given for the coupon placement within the chamber, as the rods are inserted vertically with an evident hierarchy in coupon position in relation to the source of EO vapour. Thus, initial experiments aimed to address concerns regarding this by treating coupon position as an independent variable and analysing results to assess any significant differences in CFU mL^{-1}. Findings from this study indicated that in both untreated and treated samples, log reduction in CFU mL^{-1} was not significantly different, regardless of whether the coupon was at the top, middle or bottom and thus, a conclusion can be drawn that coupon position is no longer a factor that needs considering in this model. Other general notes

regarding the design of the vapour chamber may consider the possibility for “leaks”. Whilst a concerted effort was made to ensure the rods were fitted snuggly, and the lid/base had intact seals, this was not an issue that was focussed on. In fact, this research welcomed the occurrence of minor escaping of vapour as this would represent that of real-life scenarios where this method of application may be used.

In the previous chapter of this study, evidence showed that a concentration of 0.2% (v/v) cinnamon bark EO in broth dilution was capable of producing >3 log reductions in biofilms grown on polycarbonate coupons, after 24 h contact, and at a concentration of 2% (v/v), greater than 8 log reductions within 10 minutes of contact time were observed. Whilst it is difficult to make a direct comparison of broth dilution concentrations to cinnamon bark EO in vapour form, volumes of EO used in the base of the vapour chamber were chosen to represent practical volumes, which kept cost in mind, though also reflected comparable concentrations.

Results from the coupon position experiment were collated to represent 24 hour testing of biofilms with 0.2 µL cm^{-3} EO vapour. This revealed a significant 5.60 log reduction of biofilms after this contact time ($P<0.0001$). Further testing to include lower contact times led to findings showing minimal log reduction after 10, 30 and 60 min, but a 7.50 and 7.76 log reduction after 6 h and 12 h, respectively, the latter representing a 100% reduction in CFU mL^{-1}. These results, in combination with the results from 24 h, indicate an increase in observed biofilm growth after 12 h exposure and the possibility that *P. aeruginosa* showed tolerance to the EO vapour. To investigate this further, colonies from the 24 h contact point of this experiment were subcultured, used

to inoculate the CBR and grow mature biofilms, and re-tested in the vapour chamber for 6 to 24 h using the same concentration of EO vapour. This strain was called tolerance subculture 1 (TSC-1) and results showed that TSC-1 were less susceptible to the vapour than their original counterpart. Whilst the original biofilms were reduced to less than 1.5 log CFU mL^{-1} after 6 h, TSC-1 did not reduce to less than 3 log CFU mL^{-1} throughout the testing. This was also mirrored in further testing carried out on a subculture of TSC-1 (i.e. TSC-2). Thus, this indicates that reduction by the vapour was still moderately successful, in that it achieved a >4 log reduction, the effect was markedly different from the original testing and further supports the hypothesis that *P. aeruginosa* biofilms exhibited tolerance to cinnamon bark EO. This is supported by studies that have reported *P. aeruginosa* to show intrinsic tolerance to both EOs as a whole and EO components. Cox and Markham (2007) found that *P. aeruginosa* exhibited intrinsic tolerance to eugenol, linalool, geraniol, *a*-terpineol and citral, and may have an energy dependent multidrug resistance efflux pump system. MexAB-OprM is an efflux system driven by ATP that is reported to be involved in *P. aeruginosa* tolerance to hydrophobic solvents such as hexane and *p*-xylene (Li, Zhang, *et al.* 1998). These reports could explain the tolerance that is being observed in results of this study, though further research is required to corroborate this. In contrast, Becerril *et al* (2012) found that cinnamon oil did not cause an increase in MIC in any of the 48 isolates they studied, nor did it induce any tolerance in *P. aeruginosa* following 50 passages of EO treatment. Despite these findings, it is evident that cinnamon bark EO vapour is capable of reducing the viability of preformed mature biofilms at low concentrations. Although current published

literature surrounding the effects of EO vapour on biofilms is limited, similar findings have been seen for cinnamon EO direct contact treatment of biofilms (chapter 5) and in work investigating cinnamon EO vapour against planktonic bacteria. For example, Utchariyakiat *et al.* (2016) found cinnamon bark vapour to be effective against *P. aeruginosa* at a 0.5mg/L of air concentration, which is a very low concentration. Inouye *et al* (2001) found that cinnamon bark EO vapour had a minimum inhibitory dose range of 1.56 – 12.5 mg/L air against a range of Gram-negative and Gram-positive bacteria. Křůmal *et al.* (2015) conducted a study investigating the antimicrobial activity of EOs placed inside a desiccator with planktonic bacteria and found that 5 mL of cinnamon EO (leaf and bark mix) reduced bacterial numbers by 5 log in 3 days. These findings, and the findings presented in this chapter, are in contrast to research carried out by Goñi *et al* (2009) and López *et al* (2007) who found that cinnamon EO vapour was not effective against *P. aeruginosa,* however, neither of these studies indicate if the EO was leaf or bark origin and discussions in chapter 4 have already outlined the importance of this detail, highlighting the inferior antimicrobial activity of leaf oil compared to bark oil.

Interestingly, in the present study, when volume of oil used in the chamber was increased, the treated biofilms were less susceptible to the vapour compared to those treated with a lower concentration of EO vapour. The log reductions seen for biofilms exposed to 0.2 µL cm^{-3} EO vapour was significantly lower ($P<0.01$) than that of log reductions of biofilms exposed to 2 µL cm^{-3} vapour. This dose effect is especially obvious at 6 hour and 12 hour time points where biofilms treated with 0.2 µL cm^{-3} were more reduced, if not eradicated completely, compared to biofilms treated with 2 µL cm^{-3} vapour

which showed growth throughout testing. Without further investigations it is difficult to speculate as to why this may be occurring, though comment can be made on the volatility of components within the oil. Up to 300 volatile compounds have been reported to be found in essential oil from the cinnamon plant (Kazemi and Mokhtariniya 2016) and these components can be found in trace amounts or comprise up to 90% amount of the EO (Senatore 2002, Nabavi *et al.* 2015). In liquid phase, the proportions of these components are found to be fairly stable (Reyes-Jurado *et al.* 2019). However, because each compound has a different vapour pressure and thus a varying volatility, the volatile compounds will disperse at different rates until an equilibrium is reached in a closed environment (Kloucek *et al.* 2012). Cinnamaldehyde is known to be the main constituent of cinnamon bark EO and is reported to be responsible for the antimicrobial activity of this oil (Nabavi *et al.* 2015) and within the oil presented in this study, represents 85% (chapter 4). More investigations are required to assess the composition of the vapour chamber headspace at these different vapour concentrations, which would potentially shed light on the arrangement of volatile compounds in this space and give a possible explanation for the differences seen in efficacy.

Concentrations of vapour used in this chapter were loosely based on the concentrations of liquid cinnamon bark EO used to treat biofilms in the previous chapter. In the previous chapter, concentrations 0.02, 0.2 and 2% (v/v) were used and thus, by using a crude conversion of v/v to µL per L of air to represent a vapour concentration in percentage, the same concentrations were used here, with the exception of the higher concentration as this would have been an inappropriate volume of oil at the bottom of the chamber.

However, to ensure accurate depiction of the concentration of oil in vapour treatment, concentrations were reported as µL cm^{-3}. The previous chapter (Chapter 5) identified differences when treating biofilms grown on PC coupons compared to those grown on SS coupons. Although the results of the 0.2 µL cm^{-3} vapour treatment of biofilms agreed to the previous findings, as reductions were greater for PC biofilms than SS biofilms, the same was not observed in the 2 µL cm^{-3} vapour treated biofilms. This observation may also be in connection with the possible differences in vapour phase antimicrobials at the different concentrations.

Another notable outcome of this research is that, if a conversion of the vapour concentration is carried out to translate 2 µL cm^{-3} to µL per L of air and thus percentage EO vapour, 0.2 µL cm^{-3} would be expressed as 0.02% (v/v). This concentration is up to 100-fold less than concentrations used in the direct treatment experiments of chapter 5, though was capable of achieving comparable log reductions in preformed biofilms compared to higher concentrations used in liquid application. A similar phenomenon was seen in work by Tyagi and Malik (2010b) who reported in their studies assessing activity of lemongrass EO against *Candida albicans* that lemongrass EO was much more potent in vapour form compared to lemongrass EO in broth dilutions.

The US Environmental Protection Agency (EPA) defines a disinfectant as a successful antibiofilm agent when it is capable of producing a 6 log reduction of biofilm after no more than 10 min of exposure (Environmental Protection Agency 2017). Whilst the findings presented in this chapter do not advocate the replacement of current disinfection methods with cinnamon vapour, its use

may however provide additional steps in decontaminating areas prone to biofilm formation and supporting existing protocols. Current vapour decontamination protocols include hydrogen peroxide vapour/aerosolisation systems and gaseous ozone treatments (Boyce 2016). Work investigating the decontamination of various bacteria from surfaces using gaseous ozone showed a maximum log reduction of 5.05, and this study does not imply that bacteria were in biofilm state (Moat *et al.* 2009). Hems *et al.* (2005) found that gaseous ozone had no significant effect on *Enterococcus faecalis* biofilms and Marino (2018) advocates the use of gaseous ozone for longer periods of time, i.e. overnight, to achieve adequate reduction of biofilms in small spaces. Watson *et al.* (2018) found that hydrogen peroxide vapour (HPV) was able to completely inactivate biofilms, including *P. aeruginosa* biofilms, after 100 min of exposure, and Otter *et al* (2010) reported that no Gram-negative rod bacteria were cultivated from 63 sites within an intensive care unit following HPV decontamination, although this was following a 12 hour decontamination process and an overnight aeration step. The results from this chapter suggest that cinnamon bark EO vapour shows promise in the context of air and surface decontamination, especially in healthcare settings where current protocols use dangerous substances (Laird *et al.* 2012) and often require extensive decontamination times.

In summary, this chapter aimed to address the gaps in literature which have so far failed to investigate the effect of cinnamon bark EO vapour against *P. aeruginosa* biofilms, grown in non-static biofilm growth models. This chapter presents the development of a novel vapour chamber designed to assess the vapour effects of EOs and similar compounds on biofilm viability. The CBR is

an established model for investigating biofilm growth *in vitro*, therefore this chamber was adapted to be used in conjunction with the CBR to assess the effects of vapour exposure to biofilms grown using it. Initial experiments using this vapour chamber assessed the setup and whether its design affected the application of EO vapour to the biofilms. It was found that the position of the coupon did not influence the effect of the EO vapour and thus the vapour chamber was suitable for further testing of vapour against biofilms. Cinnamon vapour at 0.2 µL cm^{-3} produced a complete reduction of biofilm after 12 h of contact, though 24 h contact was not as effective. This occurrence was further investigated to explore the possibility for tolerance of *P. aeruginosa* to cinnamon vapour. After passages of treatment, although the bacteria were not as susceptible to the cinnamon vapour, they were still significantly reduced by >4 log CFU mL^{-1}. Interestingly, when the volume of oil in the vapour chamber was increased, there was no improvement in antibiofilm effects and efficacy was in fact reduced. Speculation may suggest that there may be differences in the equilibrium of volatile components within the chamber, but further investigations are required. Overall, cinnamon EO vapour shows promise in contributing to current cleaning processes and may aid in decontamination that utilises no touch protocols.

CHAPTER 6

6 SELECTED ION FLOW TUBE MASS SPECTROMETRY (SIFT-MS) ANALYSIS OF CINNAMON EO VAPOUR AND EFFECTS OF CINNAMON EO COMPONENTS ALONE AND IN COMBINATION

6.1 Introduction

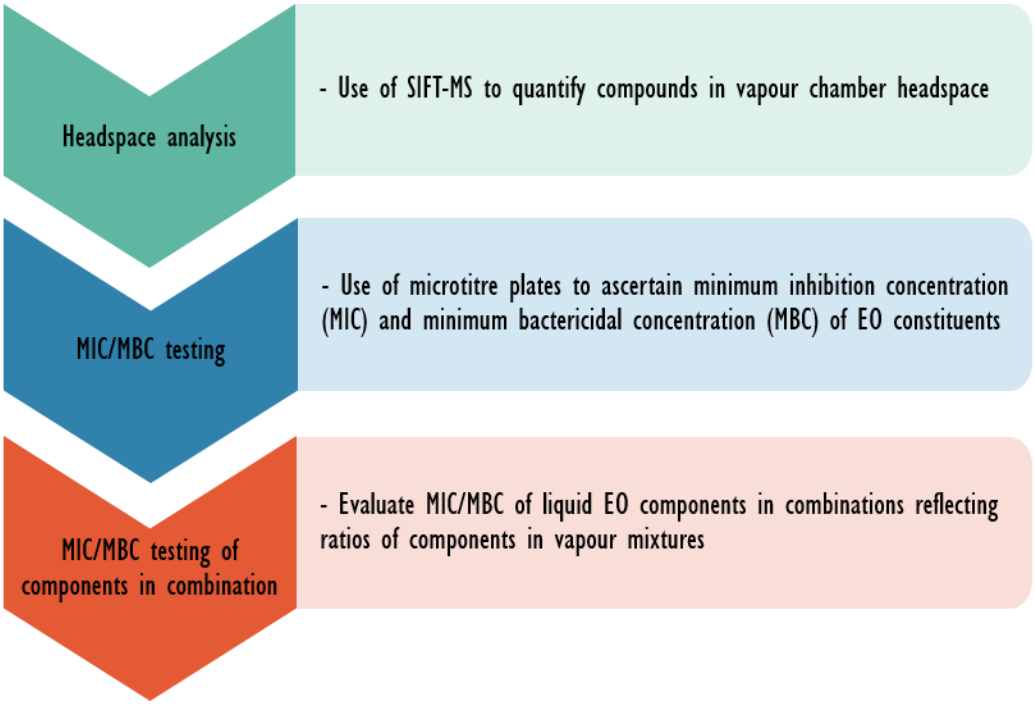
In the previous chapter (chapter 6), evaluation of the antimicrobial effects of cinnamon bark EO in vapour phase revealed that biofilms were less susceptible when EO vapour concentration was increased from 0.2 µL cm^{-3} to 2 µL cm^{-3}. It was hypothesised that the proportion of volatile components within the vapour chamber, at the different concentrations, were causing changes in anti-biofilm efficacy. The complexity of EOs, and their volatility, has already been discussed (chapter 3-6). Constituents of EOs can typically be classified into one of many compound classifications; alcohols, ethers, aldehydes, ketones, esters, amines, amides, phenols, heterocycles, and terpenes (Dhifi *et al.* 2016). Each of these constituents have a different vapour pressure, which can be used to indicate the rate of evaporation of a liquid, with substances that have higher vapour pressures, vaporising more readily (Speight 2019). The composition of a vapour mixture produced from an EO will be dependent on the vapour pressure of each constituent in the liquid phase (Guenther 1948).

The composition of cinnamon bark EO in liquid phase was analysed using gas chromatography mass spectrometry (GC-MS) in chapter 3 which identified 6 main compounds including cinnamaldehyde, limonene, eucalyptol, eugenol, benzyl benzoate and linalool. Analysis of the gaseous headspace that

cinnamon EO generates can be used to quantify these identified compounds. Selected ion flow tube mass spectrometry (SIFT-MS) is an analysis technique that has already been utilised in this thesis (chapter 3), and is a form of direct mass spectrometry that utilises soft chemical ionization to produce real-time quantitative analysis of volatile compounds (Smith and Španěl 2011). Other methods that have been used to accomplish essential oil volatile analysis include solid-phase microextraction (SPME) with follow up analysis using gas chromatography mass spectrometry (GC-MS), gas chromatography with flame ionisation detection (GC-FID) and wet effluent diffusion denuder (WEDD) techniques with GC-MS analysis (Tyagi and Malik 2011, Křůmal *et al.* 2015, Stojanović *et al.* 2019). However, all of these techniques necessitate extraction of the volatiles from the methods in which they are generated, often requiring pre-concentration of compounds before analysis and/or consideration for the fibre or compound absorption material. To my knowledge, there are no other studies that utilise SIFT-MS to investigate the vapour phase of essential oils or investigate vapour composition to elucidate anti-biofilm effects *in situ*. Therefore, this chapter aims to use SIFT-MS to analyse the headspace of vapour chambers used to treat biofilms and to investigate the composition of the volatile mixture produced from vaporisation of cinnamon bark EO within these chambers. This will potentially explain the differences seen in antimicrobial activity of cinnamon bark EO vapour at varying concentrations.

6.1.1 Chapter progression

The following flow diagram depicts a summary of the progression of this chapter.



6.2 Methods

6.2.1 Vapour chamber headspace analysis

The vapour chamber setup was similar to the method described in chapter 6. Two vapour chambers were set up as previously described; one chamber contained a lower concentration (LC) of cinnamon bark EO vapour (0.2 µL cm^{-3}) and the second chamber contained a higher concentration (HC; 2 µL cm^{-3}). Sterile blank rods were used in place of rods holding active biofilms and chambers were incubated at 37°C for the duration of the experiment, with the exception of removal from incubation during sampling. Samples were taken at 10, 30 and 60 min, and 6, 12 and 24 h. Rods were not removed at these time

points as sampling using the SIFT-MS instrument mimicked the removal of rods at these times. SIFT-MS was used to analyse the headspace of vapour chambers using methods adapted from Slade et al. (2017). Briefly, chambers were set up as previously described in chapter 6, and the headspace of the chamber was analysed using SIFT-MS (Instrument Science Limited, UK) in 'Selected Ion' mode to quantify headspace concentration of cinnamaldehyde, limonene, benzyl benzoate, eucalyptol, eugenol and linalool. Three precursor ions of H_3O^+, NO^+ and O_2^+ were used. The silicon septum of the vapour chamber was wiped with 70% ethanol before being pierced with a sterile needle attached to the SIFT-MS direct sampling inlet. The sample was vented using another sterile needle attached to a 0.2 µm syringe filter (Sartorious Stedim Biotech, Germany) which allowed headspace gases to flow freely. The SIFT-MS instrument was run for 5 seconds to allow for settling of gases and then an average part per billion (ppb) of analytes was calculated over 10 seconds of readings. The experiment was carried out independently in triplicate and results are express as average ppb.

6.2.2 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of cinnamon bark essential oil components

Results from SIFT-MS analysis of the vapour chambers (section 7.2.1) identified eucalyptol as a dominant compound present in the headspace of both low concentration (LC) and high concentration (HC) chambers. Therefore, eucalyptol was chosen, and its antimicrobial efficacy was compared to that of cinnamaldehyde, the dominant constituent of the condensate. The MIC and MBC was determined for eucalyptol and

cinnamaldehyde liquid components, alone and in combination, using methods described in chapter 4. Combinations of eucalyptol and cinnamaldehyde were mixed to reflect the ratios of the components at different time points within the vapour chamber headspace, based on methods and results of section 7.2.1. The ratios of cinnamaldehyde to eucalyptol used in combinations 1-6 are shown in Table 6.1. These ratios were calculated by dividing the concentration of eucalyptol present in the headspace by the concentration of cinnamaldehyde present in the headspace. Pure chemical compounds of eucalyptol (Sigma Aldrich, Dorset, UK) and cinnamaldehyde (Sigma Aldrich, Dorset, UK) were mixed reflecting the ratios described and diluted serially. The final concentration of cinnamaldehyde in combinations 1-3 ranged from 0.007% - 4% (v/v), with corresponding eucalyptol concentration ranging from 0.01% - 16.4% (v/v). Final tested concentrations of cinnamaldehyde in combinations 4-6 ranged from 0.007% - 2% (v/v), with corresponding eucalyptol concentration ranging from 0.07% - 28.75% (v/v).

Table 6.1 – EO Liquid Component Combinations

Ratios of cinnamaldehyde to eucalyptol used to produce combinations for minimum inhibitory concentration and minimum bactericidal concentration testing.

Combination	Cinnamaldehyde:eucalyptol ratio
1	1:3.55
2	1:1.95
3	1:0.87
4	1:12.71
5	1:11.29
6	1:7.3

6.2.3 Data analysis

Two-way ANOVA with Tukey's multiple comparison was used to assess significant differences between component concentrations in vapour chambers.

6.3 Results

6.3.1 SIFT-MS analysis of vapour chamber headspace

Results of SIFT-MS analysis of headspace from the vapour chamber containing lower concentration (LC) of cinnamon bark EO (0.2 µL cm^{-3}), the concentration that was most effective when looking at results from the previous chapter, is demonstrated in Figure 6.1 and Table 6.2. Sampling from the LC chamber between 10 min and 12 h revealed that eucalyptol was the dominant compound during this timeframe. Eucalyptol was significantly higher than all other compounds measured over the first 12 h of sampling ($P<0.0001$) and

represented 67% of the total measured compounds after 10 min of incubation in the LC chamber. However, after an initial significant increase in concentration ($P<0.0001$), eucalyptol concentration significantly decreased after 60 min ($P<0.0001$). In contrast to this, in the same LC chamber, cinnamaldehyde concentration increases over time and its concentration after 24 h of incubation is significantly greater than its concentration at 10 min ($P<0.0001$). At the 24 h sampling point, cinnamaldehyde is the dominant compound. Eugenol, linalool and benzyl benzoate concentrations did not significantly change over time ($P>0.005$) and limonene concentration significantly decreased over time ($P<0.0001$) in the LC chamber.

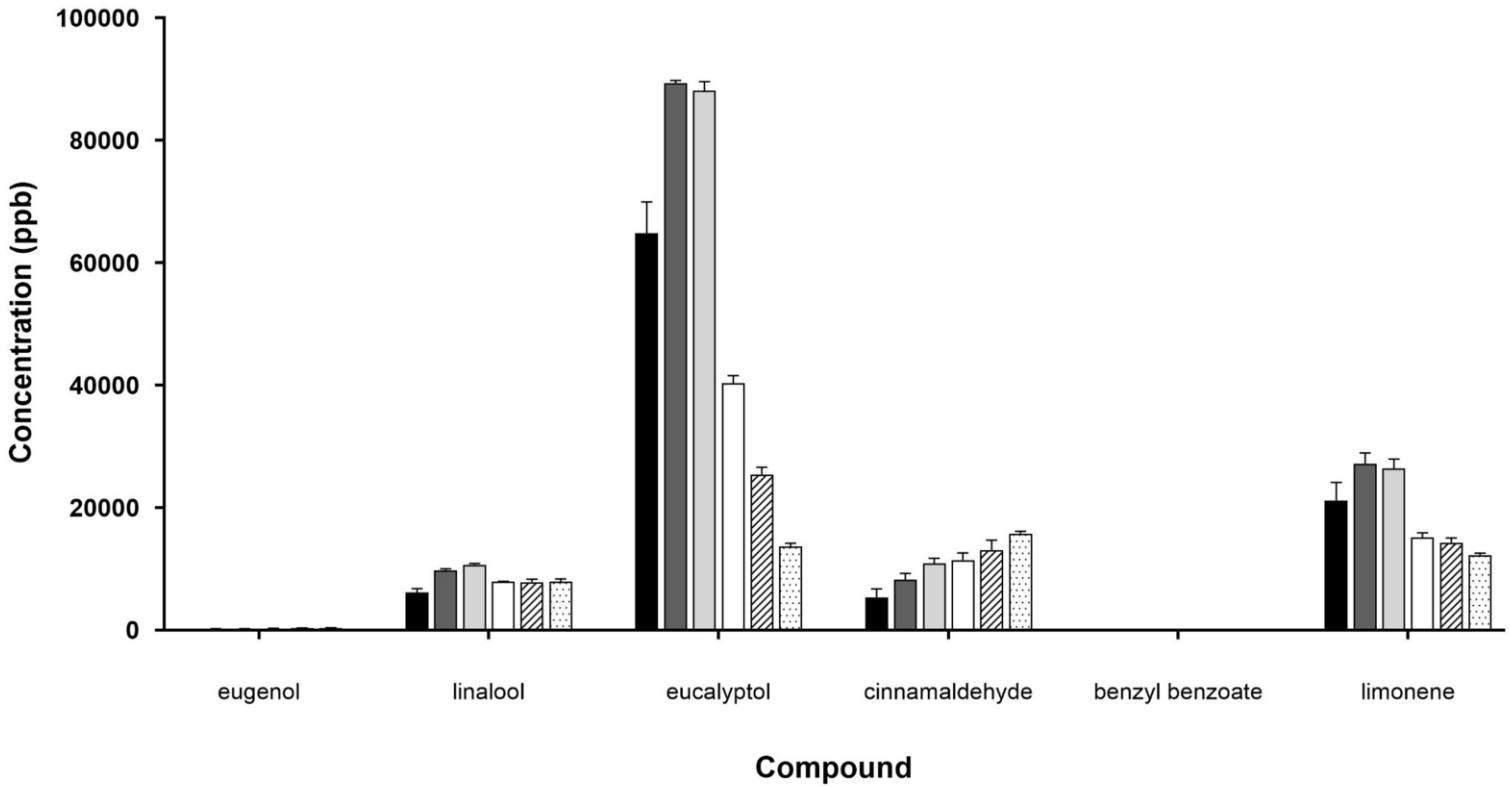


Figure 6.1 – Analysis of Cinnamon Bark EO Vapour (0.2 µL cm^{-3}).

Concentrations (ppb) of compounds present in headspace of vapour chamber containing 0.2 µL cm^{-3} cinnamon bark EO over time. ■ 10 min; ■ 30 min; ■ 60 min; □ 6 h; ▨ 12 h; ⁘ 24 h. N=3; bars show standard error.

Table 6.2 – Percentage Analysis of Cinnamon Bark EO Vapour (0.2 µL cm^{-3})

Concentration of compounds present in headspace of vapour chamber containing 0.2 µL cm^{-3} cinnamon bark EO. Values are expressed as percentage of total analytes measured by SIFT-MS ± standard error; N=3.

Time of sampling	Concentration (%)					
	Eugenol	Linalool	Eucalyptol	Cinnamaldehyde	Benzyl benzoate	Limonene
10 min	0.08±0.02	6.32±0.03	66.75±1.86	5.28±0.96	0.01±0	21.55±0.91
30 min	0.14±0.01	7.23±0.08	66.37±1.63	6.09±0.67	0.02±0	20.16±0.90
60 min	0.14±0.03	7.85±0.14	64.65±1.09	7.96±0.45	0.02±0	19.38±0.79
6 h	0.30±0.08	10.62±0.53	53.76±0.70	15.15±0.97	0.03±0	20.14±0.22
12 h	0.50±0.09	12.88±0.73	41.73±2.00	21.44±2.57	0.03±0	23.41±1.37
24 h	0.65±0.17	15.78±0.80	27.36±1.24	31.59±0.38	0.03±0	24.59±0.19
Average	0.30±0.09	10.11±1.5	53.44±6.55	14.59±4.24	0.02±0	21.54±0.84

Results of SIFT-MS analysis of headspace from vapour chamber containing higher concentration (HC) of cinnamon bark EO (2 µL cm^{-3}), the concentration that was least effective when looking at results from the previous chapter, is demonstrated in Figure 6.2 and Table 6.3. Sampling from this chamber reveals that eucalyptol remained the dominant compound over the entire 24 h sampling period. Although eucalyptol demonstrated a significant increase between 10 min and 60 min ($P<0.0001$), and then a significant decrease between 60 min and 24 h ($P<0.0001$), the initial concentration of eucalyptol at 10 min and the final concentration at 24 h were not significantly different ($P>0.005$) in the HC chamber. In the HC chamber each concentration of cinnamaldehyde measured was not significantly different from the previous time point ($P>0.05$), however the final concentration at 24 h was significantly higher than the initial concentrations at 10 min ($P<0.01$). Eugenol, linalool and benzyl benzoate concentrations did not significantly change over time ($P>0.005$) and although limonene concentration fluctuated, its concentration did not significantly change after 24 h ($P>0.05$).

A comparison of the average content of components (%) in both liquid phase and vapour phase is demonstrated in Table 6.4. In liquid phase, cinnamaldehyde represented 85.31% of total composition, though in vapour phase when cinnamon bark EO was at 0.2 µL cm^{-3} and 2 µL cm^{-3}, cinnamaldehyde represented 0.3 and 0.12%, respectively. In liquid phase, eucalyptol represented 3.82% of total composition, though in vapour phase when cinnamon bark EO was at 0.2 µL cm^{-3} and 2 µL cm^{-3}, eucalyptol represented 53.44 and 68.27%, respectively.

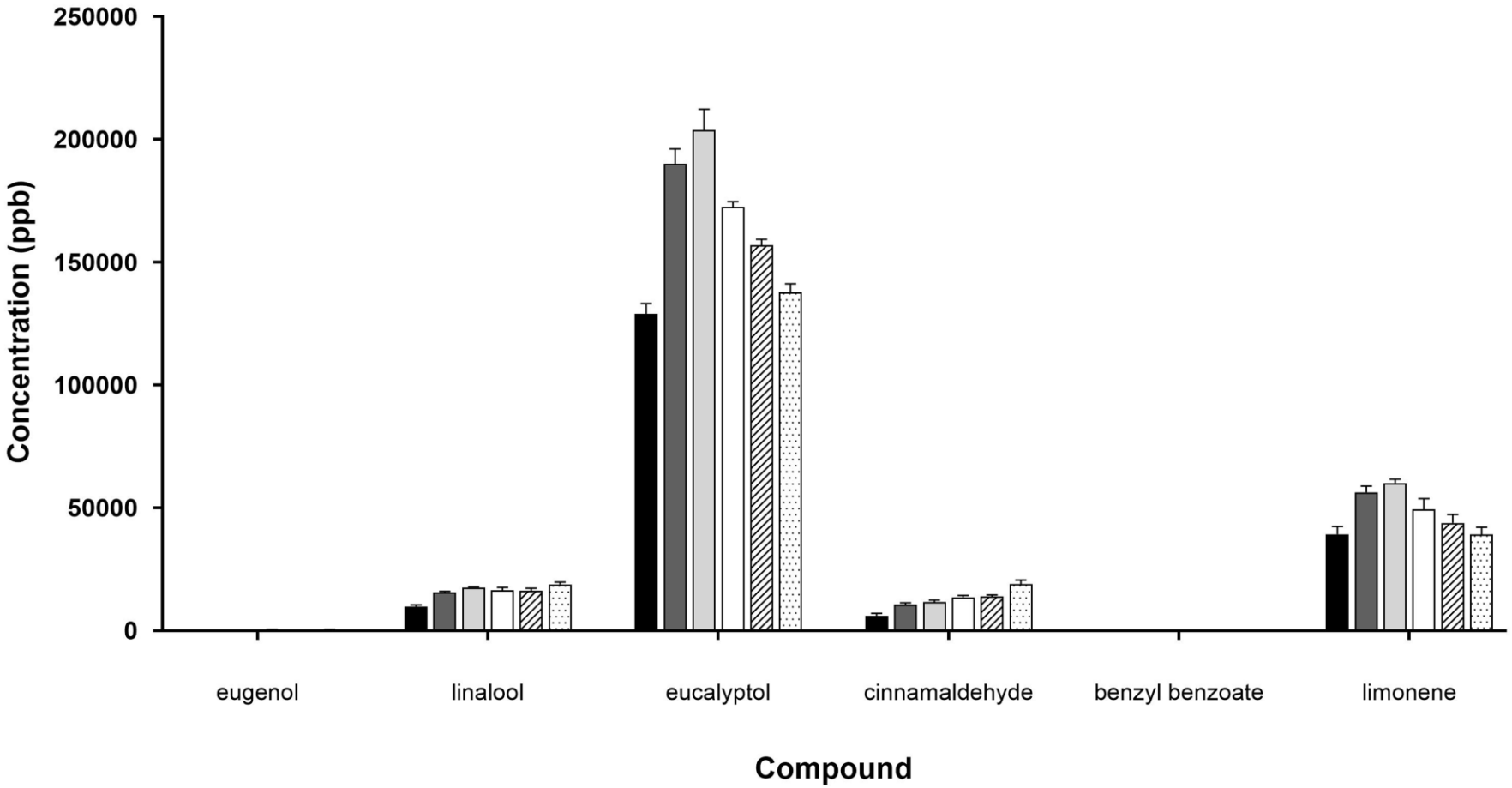


Figure 6.2 – Analysis of Cinnamon Bark EO Vapour (2 µL cm^{-3})

Concentrations (ppb) of compounds present in headspace of vapour chamber containing 2 µL cm^{-3} cinnamon bark EO over time. ■ 10 min; ■ 30 min; ■ 60 min; □ 6 h; ▨ 12 h; ⬚ 24 h. N=3; bars show standard error.

Table 6.3 – Percentage Analysis of Cinnamon Bark EO Vapour (2 µL cm^{-3})

Concentration of compounds present in headspace of vapour chamber containing 2 µL cm^{-3} cinnamon bark EO. Values are expressed as percentage of total analytes measured by SIFT-MS ± standard error; N=3.

Time of sampling	Concentration (%)					
	Eugenol	Linalool	Eucalyptol	Cinnamaldehyde	Benzyl benzoate	Limonene
10 min	0.06±0.02	5.31±0.17	70.11±1.49	3.24±0.44	0.01±0	21.26±0.87
30 min	0.09±0.02	5.71±0.18	69.68±1.61	3.87±0.33	0.02±0	20.64±1.09
60 min	0.10±0.01	5.98±0.13	69.41±1.37	3.98±0.33	0.02±0	20.52±0.94
6 h	0.15±0.01	6.48±0.32	68.46±1.84	5.38±0.18	0.02±0	19.50±1.34
12 h	0.15±0.01	7.02±0.35	67.87±1.78	6.01±0.19	0.02±0	18.93±1.27
24 h	0.17±0.03	8.76±0.30	64.09±2.07	8.78±0.64	0.03±0	18.18±1.11
Average	0.12±0.02	6.54±0.51	68.2±0.90	5.21±0.83	0.02±0	19.84±0.48

Table 6.4 – Cinnamon Bark EO Composition of Constituents

Composition comparison of components in cinnamon bark EO liquid phase, and vapour phase at different concentrations. N=3

Component	Percentage (%) of cinnamon bark EO composition		
	Liquid phase [a]	Vapour phase (0.2 µL cm^{-3}) [b]	Vapour phase (2 µL cm^{-3}) [c]
Cinnamaldehyde	85.31	0.30	0.12
Limonene	4.67	10.11	6.54
Eucalyptol	3.82	53.44	68.27
Eugenol	3.37	14.59	5.21
Benzyl benzoate	1.94	0.02	0.02
Linalool	0.90	21.54	19.84

[a] results obtained in chapter 4

[b & c] average percentage of components measured from sampling points over 24 h

6.3.2 MIC and MBC of liquid components

The MIC and MBC for cinnamon bark EO components alone or in combination against *P. aeruginosa* PAO1 are demonstrated in Table 6.5. Cinnamon bark EO MIC/MBC against this bacterium was previously determined (chapter 4) and these values were included in Table 6.5 for reference. Eucalyptol possessed no antimicrobial activity at concentrations ≤30% (v/v). The MIC of cinnamaldehyde alone was 0.06% (v/v) which is lower than the MIC of the whole EO, although MBC was 0.12% (v/v) which is unchanged from cinnamon EO. Combinations 1, 5 and 6 all had MIC and MBC values comparable to that of cinnamon EO. Combinations 2 and 3 both had improved MIC values

compared to cinnamon EO and similar to that of cinnamaldehyde alone, although MBC values were unchanged. Combination 4 had an increased MIC and MBC value of 0.25% (v/v) compared to cinnamon bark EO.

Table 6.5 – Inhibitory and Bactericidal Effects of Cinnamon Bark EO and its Components

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of cinnamon bark EO, and individual compounds either alone or in combination, against *P. aeruginosa* PAO1. *N*=4

Tested EO/compound/combination	Concentration (% v/v)	
	MIC	MBC
Cinnamon bark EO[a]	0.12	0.12
Cinnamaldehyde	0.06	0.12
Eucalyptol	>30	>30
Combination 1[b]	0.12	0.12
Combination 2[c]	0.12	0.12
Combination 3[d]	0.06	0.12
Combination 4[e]	0.25	0.25
Combination 5[f]	0.12	0.12
Combination 6[g]	0.12	0.12

[a] MIC and MBC results from chapter 4

[b] 1:3.55 [c] 1:1.95 [d] 1:0.87 [e] 1:12.71 [f] 1:11.29 [g] 1:1.73; cinnamaldehyde:eucalyptol ratio used.

6.4 Discussion

The previous chapter (chapter 6) investigated the anti-biofilm effects of cinnamon bark EO vapour and results demonstrated decreased success when concentration of EO was increased within the chamber. This chapter continued to investigate these findings and did so by focusing on analysing the headspace of vapour chambers containing cinnamon bark EO at different concentrations, with the aim to elucidate why this outcome occurred. The vapour chambers were designed with a silicon port to facilitate *in situ* sampling of the headspace. Analysis was carried out using SIFT-MS, an instrument capable of identifying and quantifying volatile organic compounds (VOCs) and a method that has been described extensively elsewhere (Wang *et al.* 2002, Španěl *et al.* 2006, Smith and Španěl 2011, Lacko *et al.* 2019). Other methods such as SPME, GC-FIDD and WEDD can be time-consuming and cannot be used to easily measure constant chemical evolution of a headspace (Lacko *et al.* 2019). The advantage of using SIFT-MS is that this method does not suffer from these drawbacks, there is no need for pre-concentration or extraction of volatiles and thus, real-time *in situ* sampling is carried out without disruption of the vapour environment and experiment. After literature survey, it is to the best of my knowledge that this study is the first to use SIFT-MS to analyse EO headspace and quantify the composition of the vapour mixture produced. Whilst studies have been carried out to evaluate the vapour composition of EOs (Tyagi and Malik 2011, Křůmal *et al.* 2015, Stojanović *et al.* 2019), these studies use the methods described above, they do not assess alternative concentrations of EOs and sampling is not in the circumstance of anti-biofilm testing. Analysis of cinnamon bark EO using GC-MS in chapter 4 identified the

main components in liquid phase. These components were used in the experimental setup of SIFT-MS to identify their composition in the vapour headspace. In the lower concentration (LC) vapour chamber, containing 260 µL of cinnamon EO, SIFT-MS analysis revealed that after 10 minutes of incubation, eucalyptol was the dominant constituent. This is in contrast to the liquid phase composition of cinnamon EO which identified cinnamaldehyde as the dominant constituent. This observation was mirrored in the higher concentration (HC) vapour chamber containing 2.6 mL of EO, where eucalyptol was also dominant in the headspace. An EO vapour mixture is the result of volatile components of the EO going into gaseous state. Its composition relies on the partial vapour pressures of the EO constituents. In circumstances where a liquid is comprised of constituents possessing varying vapour pressures, components with the higher vapour pressure, and thus greater volatility, will be found in higher concentrations within the headspace (Guenther 1948). The vapour pressure of the constituents of cinnamon bark EO are demonstrated in Table 6.6. This data shows that of the 6 compounds present in cinnamon EO, eucalyptol has the highest vapour pressure which explains the dominance of eucalyptol in the vapour phase of cinnamon EO. This is comparable to results demonstrated by Křůmal *et al.* (2015) who also compared the composition of cinnamon oil (bark/leaf mix) in both liquid and gaseous states using WEDD, and found that although cinnamaldehyde was the major constituent of the liquid phase, it was no longer the main compound in vapour state. Stojanović *et al* (2019) found that in several species of *Thymus* (thyme) essential oils, the main constituent in liquid phase was not the main constituent in gaseous state and Tyagi and Malik (2011) also observed

differences in composition when comparing liquid and vapour state of eucalyptus EO, although the balance of constituents remained similar.

Table 6.6 – Vapour pressures of constituents of cinnamon bark EO (mm Hg)

Constituent	Vapour pressure (at 25±1.5 °C)	Reference
Cinnamaldehyde	0.0289 mm Hg	(Perry *et al.* 1997)
Limonene	1.51 mm Hg	(Li, Perdue, *et al.* 1998, Hoskovec *et al.* 2005)
Eucalyptol	1.90 mm Hg	(Riddick *et al.* 1986)
Eugenol	0.0221 mm Hg	(Van Roon *et al.* 2005)
Benzyl benzoate	0.000224 mm Hg	(Daubert and Danner 2013)
Linalool	0.159 mm Hg	(Li, Perdue, *et al.* 1998)

Findings presented in this study, in addition to those from the literature, highlight the importance of determining composition of both the liquid phase and vapour phase of EOs, and that for cinnamon bark EO, knowledge of the composition of the EO in liquid phase cannot be translated to composition of the same EO in vapour phase. This is supported by Leggio *et al.* (2017) who advocate the critical nature of determining the composition of EOs in gaseous phase in order to fully understand its activity.

Eucalyptol was the dominant component in both LC and HC vapour chambers, however, whilst eucalyptol stayed >64% of total components during the entire 24 h sampling period in the HC chamber, in the LC vapour chamber there was

reduction in eucalyptol and this component was no longer dominant after 24 h. The main constituent after 24 h in the LC chamber was cinnamaldehyde. This is a possible explanation for the greater antimicrobial efficacy of the LC chamber against biofilms described in chapter 6 as cinnamaldehyde is suggested to be the main antimicrobial constituent. In the HC chamber, cinnamaldehyde did not exceed 8.78% of the total composition which could also explain the inferior anti-biofilm effects of the HC chamber. Studies have reported that minor components of oil composition are often critical in the antimicrobial effects of the whole oil (Burt 2004), and although the effects of the minor components are often reported as synergistic (Goñi *et al.* 2009), there have been reports of antagonism (Gill *et al.* 2002, Mourey and Canillac 2002, Bassolé and Juliani 2012). Synergism is when combinations of antimicrobials result in an increase in antimicrobial activity that is greater than the sum of individual components, whereas antagonism is a decrease in antimicrobial activity when in combination (Chouhan *et al.* 2017). Thus, this chapter continued investigations to evaluate the antimicrobial properties of eucalyptol and cinnamaldehyde, alone and in combination, to look for synergistic or antagonistic behaviour in liquid phase when used to treat *P. aeruginosa*. Cinnamaldehyde alone had an MIC lower than that of the whole oil (Table 6.5), whereas eucalyptol possessed no antimicrobial activity at tested concentrations. Combinations of these two constituents revealed that when eucalyptol was less than cinnamaldehyde in the final mixture (i.e. combination 3), the MIC and MBC reflected the MIC and MBC values of cinnamaldehyde alone. This finding is supported by Tak and Isman (2017) who found that when eucalyptol was added to cinnamaldehyde at a ratio of

1:0.8 (cinnamaldehyde:eucalyptol), synergistic effects were observed and insecticidal activity was increased. In the present study, when the combination resulted in eucalyptol being no more than 12 times greater than cinnamaldehyde (i.e. combinations 1, 2, 5 and 6), the MIC and MBC values were comparable to the MIC/MBC value for the whole EO, and when eucalyptol was more than 12 times that of cinnamaldehyde in the mixture (i.e. combination 4), the MIC and MBC value was increased. This not only supports the literature which suggests that cinnamaldehyde exerts the majority of cinnamon bark EOs' antimicrobial effects, but these findings also indicate that eucalyptol may have an additive or antagonistic effect against cinnamaldehyde, both in liquid phase and possibly in vapour phase, depending on the ratio of the two components. Of course, it is also noted that throughout the sampling of vapour chambers at both concentrations, limonene is >18% of the composition at all sampling points. Limonene is reported to possess antimicrobial activity (van Vuuren and Viljoen 2007). Tak and Isman (2017) demonstrated that the addition of limonene to cinnamaldehyde at a ratio of 0.8 (w:w) caused synergistic activity, whilst Andrade-Ochoa *et al.* (2018) found that limonene in equal parts with cinnamaldehyde produced synergistic activity. However, to my knowledge no data is available to describe the synergistic or antagonistic effects of combining limonene with cinnamaldehyde at higher ratios. Therefore, when considering findings presented in this chapter, it is difficult to attribute the antagonistic effects observed in the data presented here to eucalyptol alone, when other minor components may be contributing. Synergy can also be evaluated using other methods including the checkerboard assay and fractional inhibitory

concentration (FIC) index, the E-test method or an immunometric checkerboard assay with fluorescent output for real-time synergy analysis (Langeveld *et al.* 2014).

In summary, despite cinnamaldehyde being reported previously in this thesis (chapter 4) as the dominant compound within cinnamon bark EO liquid, the findings from this chapter demonstrate that this is not reflected in the composition of cinnamon bark EO in vapour phase. This study highlights the importance of vapour composition analysis when the intended use of an EO is in its vapour form, as composition of the liquid phase does not always reflect the composition of the vapour. Eucalyptol is known to have the highest vapour pressure of the components identified in cinnamon bark EO used here and thus it often dominated the headspace of vapour chambers used in this study. It is suggested from findings presented here that reduced anti-biofilm effects exhibited in the vapour chamber containing a greater volume of EO is due to this dominance of eucalyptol in the headspace. It is suggested that eucalyptol may be having an antagonistic effect and inhibiting cinnamaldehyde from exerting its known antimicrobial effects. MIC and MBC testing of components alone and in combination in this chapter suggested that this is true, although further investigations are needed to evaluate the effects of other minor compounds found in cinnamon EO.

This study was the first to evaluate the headspace of vapour chambers used to treat biofilms with EO vapour and puts forth a novel method of *in situ* sampling to assess vapour composition using SIFT-MS. This method is of particular importance as this chapter has highlighted the importance of using a method that is capable of real time analysis in circumstances where vapour

composition is constantly evolving and where this varying composition of vapour is critical in its effects. The findings in this chapter suggest that use of pure components from EOs as antimicrobials may be beneficial, due to the potential interference from other minor compounds and the risk of antagonistic behaviour when part of the whole EO. However, if whole EO is to be used as an antimicrobial, in either liquid or vapour applications, then analysis must be carried out on the phase that it is intended to be used in, to fully understand which constituents are responsible.

CHAPTER 7

7 DISINFECTANT POTENTIAL OF CINNAMON BARK ESSENTIAL OIL

7.1 Introduction

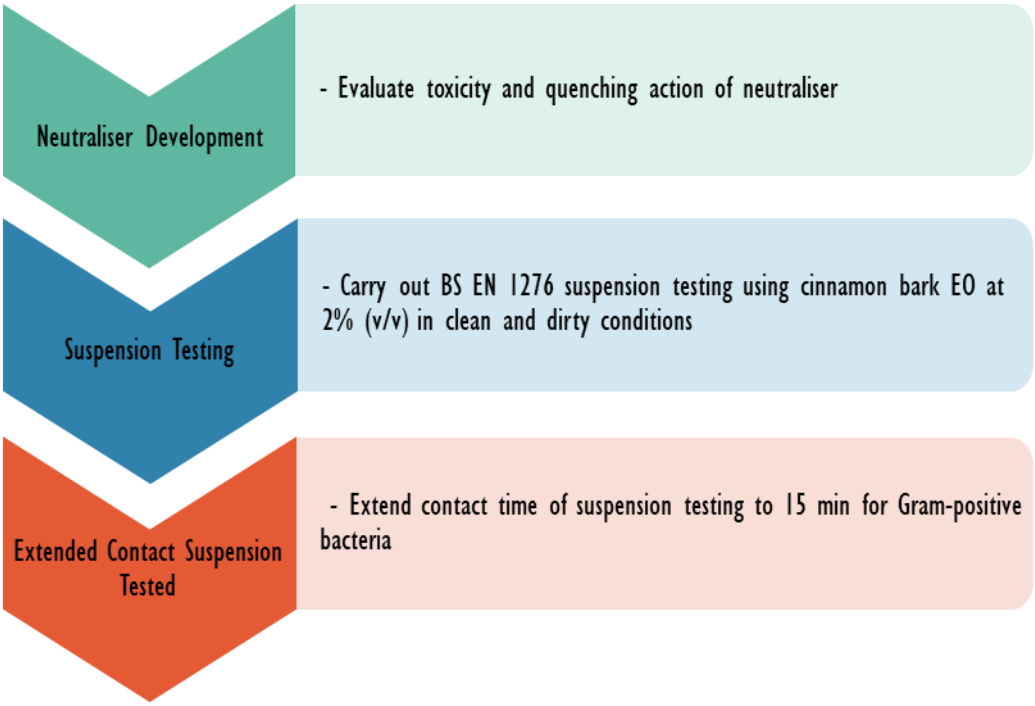
EOs as antimicrobials, and their potential applications, are well described in the literature and supported thus far in this thesis. Chapter 4 identified the broad spectrum activity of several EOs and chapters 4-6 highlighted the antimicrobial activity of cinnamon EO against *P. aeruginosa,* and its ability to cause significant reduction in bacterial viability at low concentrations following short contact times. The potential real-world application of EOs, and their use as antimicrobials, was also briefly discussed in Chapter 1. Many of the studies that look at EOs as antimicrobials have looked at their potential application in the food industry (Chouhan *et al.* 2017). EOs have been used to delay spoilage and improve aromatic qualities in vacuum packed meat and fish (Burt 2004); in antimicrobial packaging films or coatings to reduce surface molds and bacterial contamination (Rodríguez *et al.* 2007, Gómez-Estaca *et al.* 2010, Sanla-Ead *et al.* 2011, Avila-Sosa *et al.* 2012, Alvarez *et al.* 2014, Tongnuanchan and Benjakul 2014); applied to baked goods (Ju *et al.* 2018); and in aquaculture to treat fresh water fish (Kačániová *et al.* 2017). EO application has also been suggested in the cosmetic industry in mouth rinses to protect against oral bacteria (Moon *et al.* 2011); as a topical antimicrobial (May *et al.* 2000); and as wound disinfectants (Warnke *et al.* 2009). EOs have also been suggested for use as disinfectants (Laird *et al.* 2012). A disinfectant is an antimicrobial capable of interrupting an infection on contaminated inanimate non-living objects (Presterl *et al.* 2019). In developing a new

disinfectant the British standards (BS EN) employ a 3 phase testing regimen which first uses quantitative suspension tests without regard to specific application (Gebel *et al.* 2013). This is followed by quantitative tests in both suspensions and simulated practical testing within the laboratory, in conditions representative of intended use; and finally field tests are carried out under practical conditions (Gebel *et al.* 2013). Under European standards, BS EN 1040 covers phase 1, and BS EN 1276 and BS EN 13697 covers phase 2, for development of an antibacterial disinfectant intended for food, industrial, cosmetic and institutional areas (British Standards Institution 2009, 2015). Phase 3 standards are yet to be developed for disinfectant testing (Sandle 2017). There are very few studies to date that investigate the potential of EOs under these European standards of disinfection. Yangui *et al.* (2009) studied the bactericidal and fungicidal activities of *Pituranthos chloranthus* EOs and to study their potential use as powerful and natural disinfectant; Bouaziz *et al.* (2009) looked at EOs from *Salvia officinalis;* Messager *et al.* (2005) assesses the antibacterial activity of tea tree oil and tea tree oil containing products; and Falcó *et al.* (2019) evaluated the sanitizing effects of 9 EOs.

To my knowledge there is currently no research that uses the European standard BS EN 1276 to investigate cinnamon bark EO or its disinfection potential compliant with these standards. Therefore, the aim of this chapter is to evaluate cinnamon bark EO as a potential disinfectant against a panel of bacteria described in the BS EN 1276 standard.

7.1.1 Chapter progression

The following flow diagram depicts a summary of the progression of this chapter.



7.2 Methods

7.2.1 Media and reagents

7.2.1.1 Diluent

Diluent comprised of 8.5 g L^{-1} sodium chloride and 1 g L^{-1} tryptone pancreatic digest of casein, at pH 7 and was sterilised by autoclave.

7.2.1.2 Disinfectant product

Cinnamon bark EO was diluted in water containing 0.5% (v/v) Tween 80 to achieve a concentration of 2.5% (v/v). This solution was confirmed to stay in a stable homogenous suspension for the duration of >2 h. The concentration of EO was prepared at 1.25x final concentration to achieve 1x concentration in test method. Disinfectant product was used within 1 h.

7.2.1.3 Interfering substance

Bovine serum albumin (BSA; Sigma Aldrich, Dorset, UK) was used as an interfering substance as recommended by BS EN 1276. Testing was carried out under both clean conditions (0.3 g L^{-1} BSA) and dirty conditions (3 g L^{-1}). BSA stock solutions were made up at 10x concentration, filter sterilised with 0.2 µM filter and kept at 4°C until needed. Stock solutions were used within 1 month.

7.2.1.4 Neutraliser

A neutralising solution was based on the aldehyde neutraliser composition recommended by BS EN 1276. Tween 80 (30 g L^{-1}), lecithin (3 g L^{-1}) and L-histidine (1 g L^{-1}) were added to phosphate buffer (34 g L^{-1} monopotassium phosphate, pH 7.2) and sterilised by autoclaving. Neutraliser controls were carried out to assess its toxicity against the test bacteria and its antimicrobial quenching activity. All neutraliser validation testing was carried out at 20°C.

7.2.2 Test organisms and culture preparation

Test organism used in this chapter included *Staphylococcus aureus* (NCTC 12981), *Pseudomonas aeruginosa* (ATCC 15442), *Escherichia coli* (ATCC

10536) and *Enterococcus hirae* (ATCC 10541). Cultures were prepared as described in BS EN 1276:2009.

7.2.2.1 Preparation of working culture

Frozen stocks of organisms were revived by streaking onto TSA agar plates and incubating at 37°C for 24 h. After 24 h, a second subculture streak plate was prepared in the same way. These plates were the working cultures from which suspensions were made in broth.

7.2.2.2 Test suspension

For all organisms a test suspension was prepared by taking loopfuls of working culture (section 7.2.1.1) and placing in 10 mL of diluent, in 100 mL flasks. The flasks were vortexed for 1 min. This suspension was then adjusted using diluent and a spectrophotometer at OD_{620nm}, until number of cells was approximately 1.5-5 x 10^8 CFU mL^{-1}. Suspensions were used within 2 h.

7.2.2.3 Validation suspension

A validation suspension was prepared from the test suspension (section 7.2.1.2). A 10^{-5} dilution of test suspension was diluted 1:3 in diluent to achieve approximately 3.0 x 10^2 to 1.6 x 10^3 CFU mL^{-1}. Suspensions were used within 2 h.

7.2.3 Neutraliser validation

Two validation tests were performed in parallel to testing to ensure the neutraliser used did not exert any lethal effects on that bacteria tested, and to

ensure that it quenched the antimicrobial activity of the disinfectant after the required contact time. Validation was deemed successful if ≥50% of validation suspension bacteria were viable following testing, as described by BS EN 1276.

7.2.3.1 Neutraliser toxicity

To ensure that the neutraliser had no bactericidal effects against test bacteria, 8 mL of neutraliser was added to 1 mL of water and 1 mL of validation suspension. After 5 minutes of incubation, the solution was serially diluted in sterile PBS and CFU mL^{-1} was calculated by spiral plating onto TSA and incubating for 24 h at 37°C. Toxicity testing was carried out alongside each replicate of suspension testing (N=3).

7.2.3.2 Neutraliser quenching

To ensure that the neutraliser sufficiently quenched the disinfectant product 1 mL water was mixed with 1 mL diluent, followed by the addition of 8 mL disinfectant product. After 1 min of incubation, 1 mL of solution was removed and added to 8 mL of neutraliser. Following a 5 min incubation period, 1 mL of validation suspension was added, and the solution was left for 30 min, followed by enumeration by spiral plating onto TSA and incubating for 24 h at 37°C. Quenching testing was carried out alongside each replicate of suspension testing (N=3).

7.2.4 Suspension testing

Suspension testing was carried out under both clean and dirty conditions. One millilitre of interfering substance was mixed with 1 mL of test suspension. Following a 2 min incubation, 8 mL of disinfectant product (2.5% v/v) was added, and mixture was shaken. Final concentration of disinfectant was 2% (v/v). After 5 min of contact, 1 mL was removed and transferred to a tube containing 8 mL of neutralizer and 1 mL of water. After 5 min, the solution was serially diluted, and bacteria were enumerated on TSA using a spiral plater. Plates were incubated for 24 h at 37°C. As described by BS EN 1276, a disinfectant is deemed successful when a 5 log reduction is achieved within 5 min of contact time.

A control test was carried out at the same time as suspension testing using validation suspension, and water in place of disinfectant product, to verify that suspension testing had no lethal effects on the bacteria. The control was deemed successful if ≥50% of validation suspension bacteria were viable following testing, as described by BS EN 1276. Each experiment had three replicates and were carried out independently in triplicate.

7.2.5 Extended contact suspension testing

Extended contact time testing was carried out for Gram-positive bacteria; *Staphylococcus aureus* (NCTC 12981), and *Enterococcus hirae* (ATCC 10541). The method was carried out as described in section 7.2.4, however, disinfectant product was left for a total of 15 min contact time before solution was neutralised. Each experiment had three replicates and were carried out independently in triplicate.

7.2.6 Data analysis

Two-way ANOVA with Dunnett's multiple comparisons were used to assess significant differences in validation testing, whilst Two-way ANOVA was used to evaluate significant differences in suspension testing.

7.3 Results

7.3.1 Neutraliser toxicity

Results from validation of neutraliser toxicity are shown in Table 7.1. The neutraliser did not reduce the bacteria tested significantly ($P>0.05$) and did not reduce the validation suspension by more than 50%, confirming that the neutraliser was not toxic.

7.3.2 Neutraliser quenching

Results from validation of neutraliser quenching are shown in Table 7.2. The neutraliser did not significantly reduce the bacteria tested ($P>0.05$), in either clean or dirty conditions. The neutraliser did not reduce organisms more than 50%, confirming that the neutraliser was able to efficiently quench the effects of cinnamon bark EO.

Table 7.1 – Neutraliser Toxicity Testing

Results of neutraliser toxicity validation testing showing difference between original validation suspension CFU mL^{-1} and viable bacteria following 5 min contact with neutraliser. N=3; values expressed as average difference in log_{10} CFU mL^{-1} of viable bacteria ± standard error.

Bacteria	Difference in log_{10} CFU mL^{-1}	Pass/Fail[a]
S. aureus	-0.31 ±0.42	Pass
E. hirae	-0.25 ±0.45	Pass
E. coli	-0.62 ±0.45	Pass
P. aeruginosa	-0.34 ±0.43	Pass

[a] Pass defined as no more than 50% log reduction when compared to validation suspension (CFU mL^{-1}) as described by BS EN 1276.

Table 7.2 – Neutraliser Quenching Testing

Results of neutraliser quenching validation testing, in both clean and dirty conditions, showing difference between original validation suspension CFU mL^{-1} and viable bacteria following 30 min contact with neutralised disinfectant. N=3; values expressed as average difference in log_{10} CFU mL^{-1} of tested bacteria ± standard error.

	Clean		Dirty	
Bacteria	Difference in log_{10} CFU mL^{-1}	Pass/Fail[a]	Difference in log_{10} CFU mL^{-1}	Pass/Fail[a]
S. aureus	-0.65 ±0.15	Pass	-0.21 ±0.52	Pass
E. hirae	-0.55 ±0.23	Pass	-0.55 ±0.16	Pass
E. coli	-1.10 ±0.03	Pass	-1.00 ±0.17	Pass
P. aeruginosa	-0.33 ±0.33	Pass	-0.45 ±0.31	Pass

[a] Pass defined as no more than 50% log reduction when compared to validation suspension (CFU mL^{-1}) as described by BS EN 1276.

7.3.3 Suspension testing

Results from suspension testing of cinnamon bark EO (2% v/v) are shown in Figure 7.1. In both clean and dirty conditions, the control suspension was not significantly reduced ($P>0.05$), and bacterial viability was greater than 50% of the original validation suspension, confirming that the test method did not have any lethal effects against the bacteria tested. Cinnamon bark EO (2% v/v) was successful in significantly reducing *E. coli* ($P<0.01$) and *P. aeruginosa* ($P<0.05$), in both clean and dirty conditions, by >5 log CFU mL^{-1}. *E. coli* showed a log reduction of 7.06 in both clean and dirty conditions, whilst *P. aeruginosa* showed a 7.16 log reduction in both conditions. Although cinnamon EO was able to significantly reduce *S. aureus* ($P<0.001$) and *E. hirae* ($P<0.05$), it was unable to cause a greater than 5 log reduction for either of these organisms within 5 min. In clean conditions *S. aureus* was reduced 2.23 log and *E. hirae* was reduced 0.71 log, in dirty conditions *S. aureus* was reduced 1.02 log and *E. hirae* was reduced 0.46 log. BSA did not have any significant effect on antimicrobial activity ($P>0.05$), for any of the testing conditions.

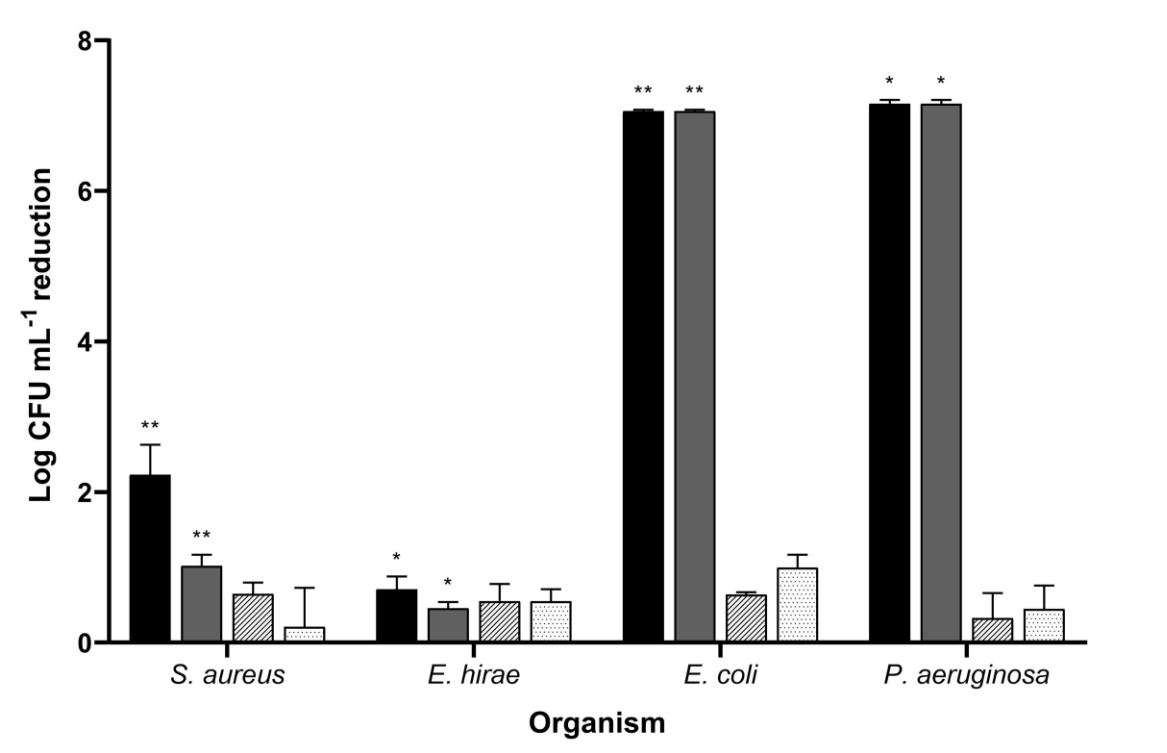


Figure 7.1 – Disinfectant Activity of Cinnamon Bark EO (2% v/v) After 5 min Contact

Average log CFU mL^{-1} reductions of bacteria after disinfection suspension testing with cinnamon bark EO (2% v/v) for 5 min and control testing. ■ clean condition disinfection; ■ dirty condition disinfection; ▨ clean condition control; ⁘ dirty condition control. N=3; bars show standard error; asterisks indicate significant difference from starting suspension.

7.3.4 Extended contact suspension testing

Results from suspension testing of cinnamon bark EO (2% v/v) are shown in Figure 7.2. In both clean and dirty conditions, the control did not significantly reduce the validation suspension ($P>0.05$), nor did it reduce bacteria by greater than 50%.

Although cinnamon EO was able to significantly reduce *S. aureus* ($P<0.001$) and *E. hirae* ($P<0.05$), it was unable to cause a greater than 5 log reduction for either of these organisms within 15 min. In clean conditions *S. aureus* was reduced 1.37 log and *E. hirae* was reduced 0.63 log, in dirty conditions *S. aureus* was reduced 1.02 log and *E. hirae* was reduced 0.49 log. BSA did not have any significant effect on antimicrobial activity ($P>0.05$), for any of the testing conditions.

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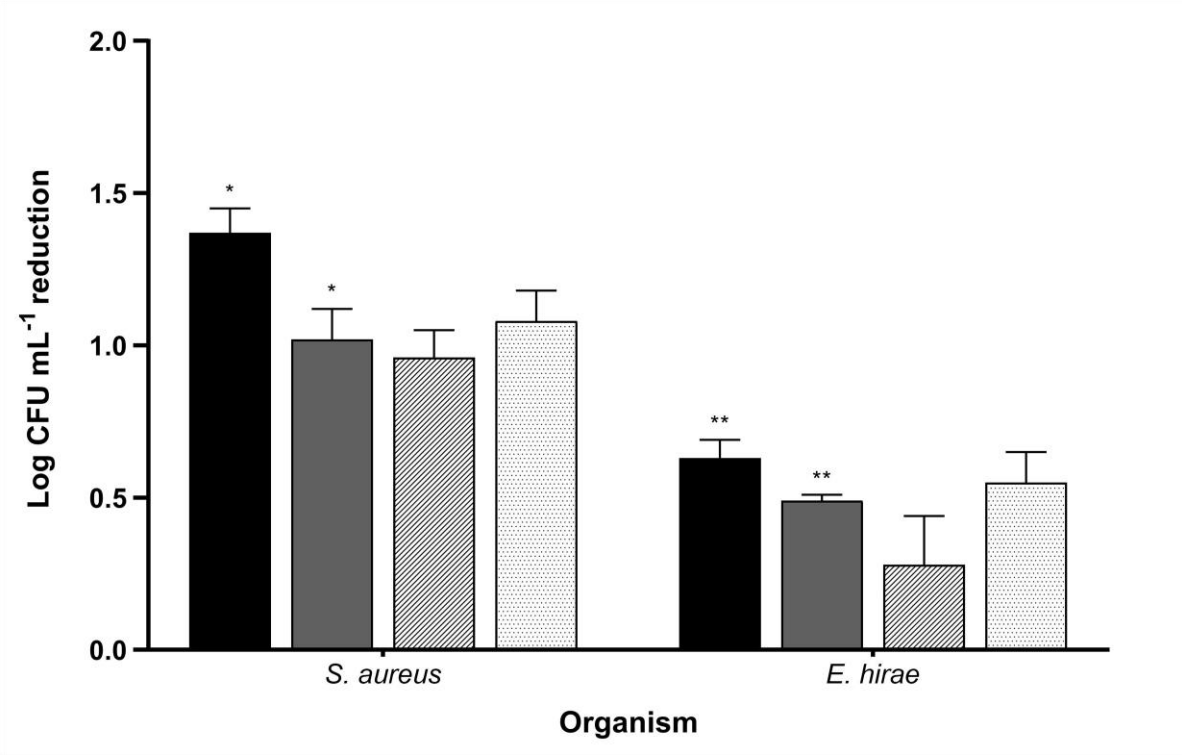

Figure 7.2 – Disinfectant Activity of Cinnamon Bark EO (2% v/v) After 15 min Contact

Average log CFU mL^{-1} reductions of Gram-positive bacteria after disinfection suspension testing with cinnamon bark EO (2% v/v) for 15 min and within control testing. ■ clean condition disinfection; ■ dirty condition disinfection; ▨ clean condition control; ⁘ dirty condition control. N=3; bars show standard error; asterisks indicate significant difference from starting suspension.

7.4 Discussion

The potential real world application of EOs have been discussed previously in this chapter introduction, and surface disinfection has been recognised as a promising application. Previous chapters thus far have investigated the antimicrobial activity of cinnamon bark EO, in liquid state, against a range of planktonic bacteria and *P. aeruginosa* biofilms. Cinnamon EO exhibited success against a broad range of both Gram-positive and Gram-negative organisms, effectively inhibiting and killing at low concentrations, and reducing bacteria by >5 log CFU mL^{-1}. However, for an antimicrobial to be considered for disinfection, it must comply with disinfection standards. Thus, this chapter aimed to investigate cinnamon bark EO as a potential disinfectant using the BS EN 1276 standards of disinfection (British Standards Institution 2009). Cinnamon EO has shown broad spectrum activity against both Gram-negative and Gram-positive bacteria (chapter 4) when using preliminary disc diffusion assays. Drawing from previous chapter investigations, cinnamon EO was tested in this chapter at a concentration of 2% (v/v), a concentration that was proven to be successful against both planktonic bacteria and biofilms (chapter 4-6). At this concentration, cinnamon EO was able to reduce *P. aeruginosa* broth cultures in time kill assays by >5 log within 2 min (chapter 4). BS EN 1276 stipulates that a successful disinfectant must achieve a 5 log reduction in bacterial load within 5 min, however, the panel of bacteria that are described includes both Gram-negative and Gram-positive bacteria.

BS EN 1276 also requires the use of interfering substances relative to the intended use of the disinfectant. As the proposed usage of cinnamon EO in this chapter was as a general purpose disinfectant to be used in food,

industrial, cosmetic and institutional areas, BSA was used as an interfering substance, as recommended by BS EN 1276. The addition of BSA provides a better representation of disinfection in the real world, as organic matter is often found as an environmental contaminant, and evaluating cleaning agents in its presence mimics practical applications (Vohra and Poxton 2011). Studies have also shown that the presence of BSA quenched the activity of antimicrobials (Bessems 1998) and offered bacteria a form of protection during disinfectant challenges (Simões *et al.* 2006). In contrast to this, the present study found that the presence of BSA, at both low (0.3 g L^{-1}) and high concentrations (3.0 g L^{-1}), did not have a significant effect ($P>0.05$) on the antimicrobial activity of cinnamon EO against any of the bacteria tested.

Cinnamon EO (2% v/v) successfully reduced *P. aeruginosa* and *E. coli* by 5 log, within 5 min contact time, however EO at this concentration was unable to produce the same effects against *S. aureus* and *E. hirae* within 5 min.

Although many studies report that EOs are more effective against Gram-positive bacteria (Zaika 1988, Bisignano *et al.* 2001, Nazzaro *et al.* 2013, Chouhan *et al.* 2017, Man *et al.* 2019), conversely, several studies have reported that EOs have superior efficacy against Gram-negative bacteria (Prabuseenivasan *et al.* 2006, Lodhia *et al.* 2009, Lopez-Romero *et al.* 2015). Furthermore, a comparable finding to those presented in this study was shown by Mayaud *et al.* (2008), who found that *Cinnamomum verum* EO successfully reduced Gram-negative bacteria, but when used to challenge Gram-positive bacteria, higher concentrations and longer contact times were required.

Yangui *et al.* (2009) also found that higher concentrations of orange EOs were required to achieve 5 log reductions in Gram-positive bacteria.

The reduced efficacy of cinnamon EO against Gram-positive bacteria, compared to that of Gram-negative bacteria, may be due to the structure of Gram-positive bacteria. Whilst Gram-negative bacteria may possess an exterior lipopolysaccharide membrane, its adjacent peptidoglycan cell wall is thin, only 7-8 nm, and lacks strength and rigidity (Shrivastava *et al.* 2007). In contrast to this, Gram-positive bacteria have a much thicker peptidoglycan cell wall which can be up to 80 nm thick, and has a 3-dimensional rigid structure (Shrivastava *et al.* 2007). It has been previously discussed (chapter 4) that the mode of action of cinnamon EO may lie in its hydrophobic characteristic. The outer membrane possessed by Gram-negative bacteria is made up of phospholipids, lipopolysaccharides and various regulatory channels (Nazzaro *et al.* 2013), a feature which usually negates the passage of hydrophobic substances due to the presence of hydrophilic porin channels (Lopez-Romero *et al.* 2015). However, in the present study, Gram-negative bacteria were not less susceptible to cinnamon EO. A possible explanation for the contradictory reports of efficacy of oils against Gram-positive and Gram-negative bacteria may be the antimicrobial constituents of the EO used. A feature of cinnamon bark EO is that its main constituent is cinnamaldehyde, as reported previously (chapter 4). Aldehydes are reported to exert bactericidal effects by disrupting lipoproteins in the outer membrane (Remmal *et al.* 1993), and penetrating the outer membrane leading to cell content disruption and leakage (Walsh *et al.* 1999), which is a likely explanation for the effects seen in this study.

To assess if contact time influenced efficacy, further investigations were carried out to challenge the Gram-positive bacteria with cinnamon EO for an extended contact time of 15 min. Despite this increased contact period, cinnamon EO was unsuccessful in reducing both *S. aureus* and *E. hirae* by >5 log CFU mL^{-1}. A possible explanation for this decreased antimicrobial activity could be the presence of Tween 80 in the disinfectant product. Tween 80 is a surfactant and was used in this study to produce an oil in water emulsion which stayed homogenous throughout testing. It is reported that this emulsion of EO and surfactant could be an antagonistic combination, as was seen by Ziani *et al.* (2011), who found decreased antimicrobial activity of tea tree oil when emulsified with Tween 80. Remmal *et al.* (1993) found that when using Tween 80 as a dispersal agent, antimicrobial activity of EOs was significantly reduced. However, in contrast to this, Lu *et al.* (2018) reports that surfactants improve the antimicrobial activity of EOs by increasing surface area, improving cell membrane interactions, promoting targeted release, and providing electrostatic interactions with the bacterial cell wall. The contradictory evidence discussed here highlights the need for further investigations regarding the interactions between EOs, and the detergents used with them, to evaluate whether they are synergistic, antagonistic or additive.

In conclusion, this chapter aimed to investigate cinnamon bark EO as a potential disinfectant and evaluate its compliance with European standard BS EN 1276. To my knowledge, this study was the first to investigate cinnamon bark EO as a disinfectant and evaluate its compliance with recognised disinfectant standards. These standards required a disinfectant at this stage of testing (phase 2, step 1) to achieve a 5 log reduction within 5 min of contact.

Cinnamon EO was compliant with this standard against Gram-negative bacteria, though was unable to sufficiently reduce *S. aureus* and *E. hirae* after 5 min contact. Furthermore, an extended contact time of 15 min did not improve the efficacy of the EO against Gram-positive organisms. Although this may not be encouraging for the potential of cinnamon EO to be a stand-alone disinfectant, the results reported here have highlighted the potential for cinnamon EO to contribute to the cleaning regimes already used and that it could prove to be an effective additional constituent in future disinfectants. Within the methods used here, several potential reasons for reduced activity have been identified, including the possible interference from surfactants within emulsions used to produce homogenous disinfectant products containing EO. It is suggested that further investigations are made to evaluate disinfectant formulations which incorporate EOs, and ensure that the full antimicrobial potential of these oils are reached without hindrance from components which constitute the disinfectant. This study has provided a preliminary investigation into the use of cinnamon EO as a disinfectant, identifying its disinfectant activity against some but not all organisms, and demonstrating that further testing is needed to include extended contact times.

CHAPTER 8

8 DISCUSSION AND FURTHER WORK

The main aim of this research was to contribute to the search for novel antimicrobials, in response to the antimicrobial resistance (AMR) crisis being faced on a global scale. Essential oils (EOs) are an underutilised natural commodity, with recognised antimicrobial activity, and due to the vast selection of these plant extracts, many of them remain under exploited. The data presented in this thesis has further contributed to the current literature that exists on EOs, and has broadened the knowledge surrounding antimicrobial efficacy, potential applications and practical methods to utilise when evaluating EOs. The research and methods presented in this thesis have a wide spreading impact on many real life applications, for example: contributing to current cleaning regimes; potentially improving the efficacy of existing antimicrobials; and improving the methods utilised to assess essential oil liquid and vapour, and other antimicrobials. The work presented in this thesis provides a sound basis for further work and continuation of research into EOs as antimicrobials. Further work could be carried out to evaluate the potential of EOs to behave synergistically when in combination with antibiotics; to fully explore their mode of action and exploit these findings to target problematic bacteria; to investigate their toxicity in humans and include them in models of biofilm growth that better mimic natural and human environments, and how the surface on which bacteria grow impacts their tolerance to antimicrobials; and to further utilise the pure compounds which compose EOs and how these isolated constituents can be utilised.

EOs are extensively used across the globe with a multitude of uses, as discussed elsewhere. At present, there are no legislations which require EO manufacturers or distributers to confirm the purity and origin of essential oils, if they are not intended for cosmetics, food or the animal nutrition market (Barbieri and Borsotto 2018). This thesis has highlighted the critical importance of analysing EOs before evaluation of their antimicrobial effects. As was demonstrated here, EOs from the same plant species, but obtained from different plant parts, can have very different compositions. It is clear from the findings of chapter 4 and 7 that analysing the composition of EOs is imperative for oils intended for antimicrobial or bioactive applications. Not only do the composition of EOs in liquid state vary from batch to batch due to a variety of factors, but the composition of the liquid state EO is not always reflected when the EO is in its vapour state. Composition of EOs can also have a significant effect on the antimicrobial efficacy of the oil. Data presented in chapter 7 found that the cinnamon EO constituent eucalyptol seemed to have an antagonistic effect on the activity of cinnamaldehyde, the suggested dominant antimicrobial constituent of cinnamon EO. These findings suggest that although the use of whole EO is often successful, some of the minor components may inhibit the antimicrobial activity of other constituents. Commercially, the intricate and complex composition of natural EOs is often challenging to regulate and difficult to synthetically reproduce. Preliminary results in this thesis indicates that cinnamaldehyde had a greater antimicrobial effect than cinnamon EO. Therefore, it is suggested that further work be carried out using isolated pure compounds from cinnamon EO, i.e. cinnamaldehyde, to evaluate its antimicrobial efficacy alone.

The novel data presented in chapter 4 support the literature which indicate the mode of action of cinnamon EO against bacteria, and that it is attributed to membrane permeation. The research presented here suggests the ability of cinnamon EO to penetrate the exterior barriers of bacteria, thus causing disruptions in the membrane from which cell contents could leak. However, this could also work to the advantage of many failing antimicrobials, which are unable to penetrate these resistant bacteria, allowing a route of entry into the cell and enabling them to exert their effects. Many studies have already looked at interactions between essential oils and current antimicrobials, and found that EOs have a synergistic effect when combined with antibiotics (Langeveld *et al.* 2014). Magi *et al.* (2015) found that when in combination with carvacrol, a component of essential oils, the MIC of erythromycin against erythromycin resistant strains of Streptococci was reduced by up to 2048-fold. Nafis *et al.* (2019) found that essential oil from *Cannabis sativa* decreased the MIC of ciprofloxacin by 2- to 64-fold and decreased the MIC of fluconazole by 16-fold when used in combination against *Candida* spp. Whilst synergy studies of *Cinnamomum* species have been published (Yang *et al.* 2017, El Atki *et al.* 2019), studies using *Cinnamomum zeylanicum* are very limited (Utchariyakiat *et al.* 2016). The promising results shown in these studies, combined with those reported in this thesis, endorses the idea of using EOs to support the current antimicrobial repertoire and combat AMR. The dramatic decrease in production of new antibiotics warrants the enhancement of existing antibiotics (Rogers *et al.* 2012). Therefore, further work is suggested to more extensively

evaluate the mode of action of cinnamon EO and investigate its potential to increase the efficacy of existing antimicrobials.

The majority of persistent and untreatable infections are due to biofilm-forming bacteria. One of the issues identified with testing for novel antimicrobials is the failure to include bacteria in their biofilm state during testing (Cookson 2005). Results from chapter 5 have shown liquid applications of cinnamon EO to be highly effective against *P. aeruginosa* biofilms, which are usually known to be robust and impenetrable to many antimicrobials. *P. aeruginosa* biofilms are commonly implicated in persistent contamination sites associated with the clinical environment. This organism is known to be found in many places that common protocols either do not eradicate, or cannot reach, and thus it is proposed that cinnamon EO could be used as an antimicrobial in many of the sites that act as a reservoir for *P. aeruginosa* biofilms. Hard to reach reservoirs of bacteria could be combatted by employing EOs in their vapour form to maintain better contact with inaccessible places. The surface on which biofilms are found is another important factor when considering anti-biofilm effects. Chapter 5 observed differences in efficacy when biofilms were grown on different surfaces, highlighting the importance of understanding the underlying mechanics of biofilm formation and how this might affect treatment of these organisms *in situ.* This difference in efficacy was also observed when treating biofilms with EO vapour in chapter 6, where biofilms grown on stainless steel were less susceptible than those grown on polycarbonate plastic. This data further highlights the importance of surfaces when considering biofilm models for antimicrobial testing. Although polycarbonate plastic and stainless steel are

surface materials commonly found in a wide range of locations, including clinical environments, further work is suggested to look more extensively at a wider range of surface materials and evaluate the impact they have on biofilm growth and anti-biofilm efficacy.

EOs are extracted from plants, usually via steam distillation, and exist in a liquid form. However, the constituents of these liquids are highly volatile and thus, EOs are often utilised in their gaseous state. Novel experiments presented in chapter 6 demonstrated that cinnamon EO vapour was capable of reducing preformed mature biofilms by >5 log CFU mL^{-1} at concentrations as low as 0.2 µL cm^{-3}. Although it is acknowledged that further toxicity testing is paramount to the use of EOs in human applications, the efficacy of cinnamon EO vapour against *P. aeruginosa* biofilms shown here is important. *P. aeruginosa* is implicated in many human diseases, especially recurrent infections arising in cystic fibrosis (CF) patients, which are often caused by biofilm-forming strains (Høiby *et al.* 2010). Treatment by inhalation of EOs has been discussed in the literature (Blake and Raissy 2018) and cinnamon bark EO has been suggested for treatment of respiratory pathogens (Singh *et al.* 1995, Inouye *et al.* 2001). However, to my knowledge, research presented in this thesis was the first of its kind to assess the efficacy of cinnamon EO vapour against a shear-flow model grown biofilm which could potentially be translated to those biofilms growing within the human respiratory system. It is an important consideration, when developing methods that are used to investigate biofilms that an appropriate model of growth is used which reflects the natural existence of bacteria. Sriramulu *et al.* (2005) developed a novel

biofilm model which mimics biofilm formation within a CF lung, and Crabbé *et al.* (2017) investigated the efficacy of antibiotics against biofilms growing in a 3-dimensional lung epithelia model. Thus, if cinnamon EO is to be considered as a potential treatment for respiratory infections, it is suggested that further work be carried out to not only examine the toxicity of the EO but also its efficacy in biofilm models which better mimic the human respiratory environment.

Potential applications of EOs include that of disinfectant production. Data presented in this thesis has shown cinnamon bark EO to be an effective antimicrobial agent capable of rapid killing at low concentrations. Chapter 8 demonstrated that cinnamon bark EO was successful at reducing the viability of Gram-negative bacteria within 5 min of contact. However, the EO at this concentration was not efficient at eliminating Gram-positive bacteria, despite testing with extended contact times of 15 min. However, not only did cinnamon EO exceed the expected levels of disinfectant action against Gram-negative bacteria, this thesis also showed that over a 24 h period, cinnamon EO possessed the ability to inhibit and kill Gram-positive bacteria at very low concentrations. Furthermore, the efficacy of cinnamon EO versus biofilm-state bacteria is clear, and it has demonstrated strong anti-biofilm activities. Therefore, there is a need to investigate the disinfectant potential of this oil further, particularly in the area of surface disinfection and with a wide panel of bacteria associated with food, clinical and non-clinical environments.

Methods used in this thesis, such as the development of the novel vapour chamber and the use of SIFT-MS instrumentation, could be applied to many different *in vitro* investigations, and should not be limited to work investigating EOs. The chamber developed in this thesis can provide the means to carrying out a plethora of biofilm treatments using any antimicrobial agent that exists in a gaseous state. It facilitates the inclusion of biofilms grown using the CBR, an important model of biofilm growth when considering anti-biofilm testing. In combination with vapour analysis methods such as SIFT-MS, it enables real-time analysis of the headspace within the chamber, and potential elucidation of the mechanisms of action of antimicrobial vapours.

Overall, this thesis has demonstrated the strong antimicrobial efficacy of cinnamon EO and warrants the continuation of investigations *in vitro,* to evaluate its effects against a broader spectrum of biofilm forming bacteria. Not only does this thesis highlight the importance of cinnamon EO, but it also strengthens the rationale to continue testing other underexploited oils that are yet to be investigated. Whilst more research is required before EOs are considered safe for clinical and *in vivo* use, the work presented here strongly suggests that work investigating the environmental application of EOs must continue. When considering AMR, the data reported in this thesis provide further evidence that EOs could prove to be a crucial addition in solutions to combat the antibiotic resistance crisis. It has addressed the need for research and development of novel antimicrobials, and end products that include cinnamon EO could help improve sanitation and hygiene.

REFERENCES

Abdallah, M., Benoliel, C., Jama, C., Drider, D., Dhulster, P., and Chihib, N.-E., 2014. Thermodynamic Prediction of Growth Temperature Dependence in the Adhesion of *Pseudomonas aeruginosa* and *Staphylococcus aureus* to Stainless Steel and Polycarbonate. *Journal of Food Protection*, 77 (7), 1116–1126.

Adams, T.B., Cohen, S.M., Doull, J., Feron, V.J., Goodman, J.I., Marnett, L.J., Munro, I.C., Portoghese, P.S., Smith, R.L., Waddell, W.J., and Wagner, B.M., 2004. The FEMA GRAS assessment of cinnamyl derivatives used as flavor ingredients. *Food and Chemical Toxicology*, 42, 157–185.

Adukwu, E.C., Allen, S.C.H., and Phillips, C.A., 2012. The anti-biofilm activity of lemongrass (*Cymbopogon flexuosus*) and grapefruit (*Citrus paradisi*) essential oils against five strains of *Staphylococcus aureus*. *Journal of Applied Microbiology*, 113 (5), 1217–1227.

Ahmed, M.N.A., 2013. Effects of escapin intermediate products (EIP-K) on biofilms of *Pseudomonas aeruginosa*. Georgia State University.

Alanis, A.J., 2005. Resistance to antibiotics: Are we in the post-antibiotic era? *Archives of Medical Research*, 36 (6), 697–705.

Ali, B., Al-Wabel, N.A., Shams, S., Ahamad, A., Khan, S.A., and Anwar, F., 2015. Essential oils used in aromatherapy: A systemic review. *Asian Pacific Journal of Tropical Biomedicine*, 5 (8), 601–611.

Alvarez, M. V., Ortega-Ramirez, L.A., Gutierrez-Pacheco, M.M., Bernal-Mercado, A.T., Rodriguez-Garcia, I., Gonzalez-Aguilar, G.A., Ponce, A., Moreira, M. del R., Roura, S.I., and Ayala-Zavala, J.F., 2014. Oregano essential oil-pectin edible films as anti-quorum sensing and food antimicrobial agents. *Frontiers in Microbiology*, 5 (DEC), 1–7.

Aminov, R.I., 2010. A brief history of the antibiotic era: Lessons learned and challenges for the future. *Frontiers in Microbiology*, 1 (DEC), 1–7.

Andrade-Ochoa, S., Sánchez-Aldana, D., Chacón-Vargas, K.F., Rivera-Chavira, B.E., Sánchez-Torres, L.E., Camacho, A.D., Nogueda-Torres, B., and Nevárez-Moorillón, G.V., 2018. Oviposition deterrent and larvicidal and pupaecidal activity of seven essential oils and their major components against *Culex quinquefasciatus* say (Diptera: *Culicidae*): Synergism–antagonism effects. *Insects*, 9 (1), 1–17.

Anomaly, J., 2019. Antibiotics and Animal Agriculture. *In*: M. Selgelid, ed. *Ethics and Antimicrobial Resistance*. New York: Springer.

Armbruster, C.R. and Parsek, M.R., 2018. New insight into the early stages of biofilm formation. *Proceedings of the National Academy of Sciences*, 115 (17), 4317–4319.

Artini, M., Patsilinakos, A., Papa, R., Boović, M., Sabatino, M., Garzoli, S., Vrenna, G., Tilotta, M., Pepi, F., Ragno, R., and Selan, L., 2018. Antimicrobial and antibiofilm activity and machine learning classification analysis of essential oils from different mediterranean plants against *Pseudomonas aeruginosa*. *Molecules*, 23 (2), 482.

Arunasri, K. and Mohan, S.V., 2019. Biofilms: Microbial Life on the Electrode Surface. *In*: S.V. Mohan, S. Varjani, and A. Pandey, eds. *Microbial Electrochemical Technology*. Elsevier B.V., 295–313.

Asbahani, A. El, Miladi, K., Badri, W., Sala, M., Addi, E.H.A., Casabianca, H., Mousadik, A. El, Hartmann, D., Jilale, A., Renaud, F.N.R., and Elaissari, A., 2015. Essential oils: From extraction to encapsulation. *International Journal of Pharmaceutics*, 483 (1–2), 220–243.

Asghari, F.B., Nikaeen, M., and Mirhendi, H., 2013. Rapid monitoring of Pseudomonas aeruginosa in hospital water systems: A key priority in prevention of nosocomial infection. *FEMS Microbiology Letters*, 343 (1), 77–81.

Aspelund, A.S., Sjöström, K., Liljequist, B.O., Mörgelin, M., Melander, E., and Påhlman, L.I., 2016. Acetic acid as a decontamination method for sink

drains in a nosocomial outbreak of metallo-β-lactamase-producing Pseudomonas aeruginosa. *Journal of Hospital Infection*, 94 (1), 13–20.

Astani, A., Reichling, J., and Schnitzler, P., 2011. Screening for antiviral activities of isolated compounds from essential oils. *Evidence-based Complementary and Alternative Medicine*, 2011, 1–7.

El Atki, Y., Aouam, I., El Kamari, F., Taroq, A., Nayme, K., Timinouni, M., Lyoussi, B., and Abdellaoui, A., 2019. Antibacterial activity of cinnamon essential oils and their synergistic potential with antibiotics. *Journal of Advanced Pharmaceutical Technology & Research*, 10 (2), 63.

Avila-Sosa, R., Palou, E., Jiménez Munguía, M.T., Nevárez-Moorillón, G.V., Navarro Cruz, A.R., and López-Malo, A., 2012. Antifungal activity by vapor contact of essential oils added to amaranth, chitosan, or starch edible films. *International Journal of Food Microbiology*, 153 (1–2), 66–72.

Ayukekbong, J.A., Ntemgwa, M., and Atabe, A.N., 2017. The threat of antimicrobial resistance in developing countries: Causes and control strategies. *Antimicrobial Resistance and Infection Control*.

Azeredo, J., Azevedo, N.F., Briandet, R., Cerca, N., Coenye, T., Costa, A.R., Desvaux, M., Di Bonaventura, G., Hébraud, M., Jaglic, Z., Kačániová, M., Knøchel, S., Lourenço, A., Mergulhão, F., Meyer, R.L., Nychas, G., Simões, M., Tresse, O., and Sternberg, C., 2017. Critical review on biofilm methods. *Critical Reviews in Microbiology*, 43 (3), 313–351.

Baker, J.H., 1984. Factors affecting the bacterial colonization of various surfaces in a river. *Canadian Journal of Microbiology*, 30 (4), 511–515.

Barbieri, C. and Borsotto, P., 2018. Essential Oils: Market and legislation. *In*: H.A. El-Shemy, ed. *Potential of Essential Oils*. InTech, 107–128.

Barnes, L.-M., Lo, M.F., Adams, M.R., and Chamberlain, A.H.L., 1999. Effect of milk proteins on adhesion of bacteria to stainless steel surfaces. *Applied and Environmental Microbiology*, 65 (10), 4543–4548.

Barreto, T.V. and Coelho, A.C.D., 2015. Distillation. *In*: *Sugarcane: Agricultural Production, Bioenergy and Ethanol*. 341–363.

Baser, K.H.C. and Buchbauer, G., 2015. *Handbook of essential oils: Science, technology and applications*. 2nd ed. Handbook of Essential Oils: Science, Technology, and Applications. Florida: Taylor & Francis.

Bassolé, I.H.N. and Juliani, H.R., 2012. Essential oils in combination and their antimicrobial properties. *Molecules*, 17 (4), 3989–4006.

Becerril, R., Nerín, C., and Gómez-Lus, R., 2012. Evaluation of Bacterial Resistance to Essential Oils and Antibiotics After Exposure to Oregano and Cinnamon Essential Oils. *Foodborne Pathogens and Disease*, 9 (8), 699–705.

Bédard, E., Prévost, M., and Déziel, E., 2016. *Pseudomonas aeruginosa* in premise plumbing of large buildings. *MicrobiologyOpen*, 5 (6), 937–956.

Benzaid, C., Belmadani, A., Djeribi, R., and Rouabhia, M., 2019. The Effects of Mentha × piperita essential oil on *C. albicans* growth, transition, biofilm formation, and the expression of secreted aspartyl proteinases genes. *Antibiotics*, 8 (1), 1–15.

Bessems, E., 1998. The effect of practical conditions on the efficacy of disinfectants. *In*: *International Biodeterioration and Biodegradation*. 177–183.

Bickers, D., Calow, P., Greim, H., Hanifin, J.M., Rogers, A.E., Saurat, J.H., Sipes, I.G., Smith, R.L., and Tagami, H., 2005. A toxicologic and dermatologic assessment of cinnamyl alcohol, cinnamaldehyde and cinnamic acid when used as fragrance ingredients. *Food and Chemical Toxicology*, 43 (6), 799–836.

Bisignano, G., Laganà, M.G., Trombetta, D., Arena, S., Nostro, A., Uccella, N., Mazzanti, G., and Saija, A., 2001. *In vitro* antibacterial activity of some aliphatic aldehydes from *Olea europaea* L. *FEMS Microbiology Letters*, 198 (1), 9–13.

Blair, J.M.A., Webber, M.A., Baylay, A.J., Ogbolu, D.O., and Piddock, L.J. V, 2015. Molecular mechanisms of antibiotic resistance. *Nature Reviews Microbiology*, 13 (1), 42–51.

Blake, K. and Raissy, H., 2018. Inhaling essential oils: Purported benefits and harms. *Pediatric Allergy, Immunology, and Pulmonology*, 30 (3), 186–188.

Boire, N.A., 2013. Essential Oils and future antibiotics: New weapons against emerging 'superbugs'? *Journal of Ancient Diseases & Preventive Remedies*, 1 (2), 1–5.

Bonomo, R.A. and Rossolini, G.M., 2008. Importance of antibiotic resistance and resistance mechanisms. *Expert Review of Anti-infective Therapy*, 6 (5), 549–550.

De Bont, J.A.M., Van Dijken, J.P., and Harder, W., 1981. Dimethyl sulphoxide and dimethyl sulphide as a carbon, sulphur and energy source for growth of Hyphomicrobium spp. *Journal of General Microbiology*, 127 (2), 315–323.

Bouaziz, M., Yangui, T., Sayadi, S., and Dhouib, A., 2009. Disinfectant properties of essential oils from *Salvia officinalis* L. cultivated in Tunisia. *Food and Chemical Toxicology*, 47 (11), 2755–2760.

Bouhdid, S., Abrini, J., Amensour, M., Zhiri, A., Espuny, M.J., and Manresa, A., 2010. Functional and ultrastructural changes in *Pseudomonas aeruginosa* and *Staphylococcus aureus* cells induced by *Cinnamomum verum* essential oil. *Journal of Applied Microbiology*, 109 (4), 1139–1149.

Bowes, K.M. and Zheljazkov, V.D., 2019. Factors affecting yields and essential oil quality of *Ocimum sanctum* L. and *Ocimum basilicum* L. cultivars. *Journal of the American Society for Horticultural Science*, 129 (6), 789–794.

Boyce, J.M., 2016. Modern technologies for improving cleaning and disinfection of environmental surfaces in hospitals. *Antimicrobial*

Resistance and Infection Control, 5 (1), 1–10.

Brahmbhatt, H., Molnar, M., Pavi, V., and Rastijać, V., 2018. Synthesis, characterization, antibacterial and antioxidant potency of N-substituted-2-sulfanylidene-1,3-thiazolidin-4-one derivatives and QSAR study. *Medicinal Chemistry*, 15.

Bravo Cadena, M., Preston, G.M., Van der Hoorn, R.A.L., Flanagan, N.A., Townley, H.E., and Thompson, I.P., 2018. Enhancing cinnamon essential oil activity by nanoparticle encapsulation to control seed pathogens. *Industrial Crops and Products*, 124, 755–764.

Brayton, C.F., 1986. Dimethyl sulfoxide (DMSO): a review. *The Cornell Veterinarian*, 76 (1), 61–90.

British Standards Institution, 2009. Chemical disinfectants and antiseptics — Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food , industrial , institutional areas — Test method and requirements (phase 2, step 1). *European Committee for Standardization*, (August), 1–48.

British Standards Institution, 2015. Chemical disinfectants and antiseptics — Quantitative non-porous surface test for the evaluation of bactericidal and/or fungicidal activity of chemical disinfectants used in food, industrial, domestic and institutional areas — Test method and requirements. *European Committee for Standardization*, 1–36.

Brnawi, W.I., Hettiarachchy, N.S., Horax, R., Kumar-Phillips, G., Seo, H.-S., and Marcy, J., 2018. Comparison of cinnamon essential oils from leaf and bark with respect to antimicrobial activity and sensory acceptability in strawberry shake. *Journal of Food Science*, 83 (2), 475–480.

Brochot, A., Guilbot, A., Haddioui, L., and Roques, C., 2017. Antibacterial, antifungal, and antiviral effects of three essential oil blends. *MicrobiologyOpen*, 6 (4), 1–6.

Brook, I., 1989. Inoculum effect. *Reviews of Infectious Diseases*, 11 (3), 361–

368.

Buckingham-Meyer, K., Goeres, D.M., and Hamilton, M.A., 2007. Comparative evaluation of biofilm disinfectant efficacy tests. *Journal of Microbiological Methods*, 70 (2), 236–244.

Buckle, J., 2015. Basic plant taxonomy, basic essential oil chemistry, extraction, biosynthesis, and analysis. *In*: J. Buckle, ed. *Clinical Aromatherapy*. Churchill Livingstone, 37–72.

Budzyńska, A., Wieckowska-Szakiel, M., Sadowska, B., Kalemba, D., and Rózalska, B., 2011. Antibiofilm activity of selected plant essential oils and their major components. *Polish Journal of Microbiology*, 60 (1), 35–41.

Burt, S., 2004. Essential oils: Their antibacterial properties and potential applications in foods - A review. *International Journal of Food Microbiology*, 94 (3), 223–253.

Butnariu, M. and Sarac, I., 2018. Essential oils from plants. *Journal of Biotechnology and Biomedical Science*, 1 (4), 35–43.

Butt, A. and Khan, A., 2015. Antibiotic resistance of bacterial biofilms. *Middle East Journal of Business*, 10 (4), 38–45.

Cai, Y., Strømme, M., and Welch, K., 2014. Bacteria viability assessment after photocatalytic treatment. *3 Biotech*, 4 (2), 149–157.

Cao, L., Si, J.Y., Liu, Y., Sun, H., Jin, W., Li, Z., Zhao, X.H., and Pan, R. Le, 2009. Essential oil composition, antimicrobial and antioxidant properties of *Mosla chinensis* Maxim. *Food Chemistry*, 115 (3), 801–805.

Carpentier, B. and O.C., 1993. Biofilms and their consequences, with particular reference to hygiene in food industry. *Journal of Applied Bacteriology*, 75 (1971), 499–511.

Carson, C.F., Mee, B.J., and Riley, T. V, 2002. Mechanism of action of *Melaleuca alternifolia* (tea tree) oil on *Staphylococcus aureus* determined by time-kill, lysis, leakage, and salt tolerance assays and electron

microscopy. *Antimicrobial Agents and Chemotherapy*, 46 (6), 1914–1920.

CDC, 2018. Biggest Threats and Data [online]. *Centre for Disease Control and Prevention*. Available from: https://www.cdc.gov/drugresistance/biggest_threats.html [Accessed 1 May 2016].

Chouhan, S., Sharma, K., and Guleria, S., 2017. Antimicrobial activity of some essential oils—Present status and future perspectives. *Medicines*, 4 (3), 58.

Ciofu, O. and Tolker-Nielsen, T., 2019. Tolerance and resistance of Pseudomonas aeruginosa biofilms to antimicrobial agents— how *P. aeruginosa* can escape antibiotics. *Frontiers in Microbiology*, 10 (May).

Clark, S., 2009. Handling, safety and practical applications for use of essential oils. *In*: S. Clark, ed. *Essential Chemistry for Aromatherapy*. London: Elsevier, 231–264.

Clinical and Laboratory Standards Institute, 1999. M26-A: Methods for determining bactericidal activity of antimicrobial agents; approved guideline. *Clinical and Laboratory Standards Institute*, 19 (18).

Clinical and Laboratory Standards Institute, 2012. M07-A9: Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved Standard—Ninth Edition. *Clinical and Laboratory Standards Institute*, 32 (2), 1–88.

Clinical and Laboratory Standards Institute, 2015. M02-A12: Performance standards for antimicrobial disk susceptibility tests; approved standard—twelfth edition. *Clinical and Laboratory Standards Institute*, 35 (M02-A12), 73.

Coenye, T. and Nelis, H.J., 2010. *In vitro* and *in vivo* model systems to study microbial biofilm formation. *Journal of Microbiological Methods*, 83 (2), 89–105.

Connolly, M., Axtell, A., Hickey, S., Whalen, A., McNamara, L., Albright, D., Friedstat, J., and Goverman, J., 2017. Chemical burn From cinnamon oil. *Eplasty*, 17, ic11.

Cookson, B., 2005. A review: Clinical significance of emergence of bacterial antimicrobial resistance in the hospital environment. *Journal of Applied Microbiology*, 99 (5), 989–996.

Cortés, M.E., Bonilla, J.C., and Sinisterra, R.D., 2011. Biofilm formation, control and novel strategies for eradication. *Science against microbial pathogens: communicating current research and technological advances*, 896–905.

Courtenay, M., Castro-Sanchez, E., Fitzpatrick, M., Gallagher, R., Lim, R., and Morris, G., 2019. Tackling antimicrobial resistance 2019–2024 – The UK's five-year national action plan. *Journal of Hospital Infection*, 101 (4), 426–427.

Cowan, M.M., 1999. Plant products as antimicrobial agents. *Clinical Microbiology Reviews*, 12 (4), 564–82.

Cox, S.D. and Markham, J.L., 2007. Susceptibility and intrinsic tolerance of *Pseudomonas aeruginosa* to selected plant volatile compounds. *Journal of Applied Microbiology*, 103 (4), 930–936.

Crabbé, A., Liu, Y., Matthijs, N., Rigole, P., De La Fuente-Nùñez, C., Davis, R., Ledesma, M.A., Sarker, S., Van Houdt, R., Hancock, R.E.W., Coenye, T., and Nickerson, C.A., 2017. Antimicrobial efficacy against Pseudomonas aeruginosa biofilm formation in a three-dimensional lung epithelial model and the influence of fetal bovine serum. *Scientific Reports*, 7 (March), 1–13.

Cui, H., Li, W., Li, C., Vittayapadung, S., and Lin, L., 2016. Liposome containing cinnamon oil with antibacterial activity against methicillin-resistant *Staphylococcus aureus* biofilm. *Biofouling*, 32 (2), 215–225.

Daubert, T.E. and Danner, R.P., 2013. Physical and thermodynamic

properties of pure chemicals: data compilation. *Choice Reviews Online*, 27 (06), 27-3319-27–3319.

Davies, D.G., 2011. Biofilm dispersion. *In*: H.-C. Flemming, J. Wingender, and U. Szewzyk, eds. *Biofilm highlights*. London: Springer, 1–28.

Debabov, D., 2013. Antibiotic resistance: Origins, mechanisms, approaches to counter. *Applied Biochemistry and Microbiology*, 49 (8), 665–671.

Department of Health, 2016. Antimicrobial resistance empirical and statistical evidence-base. *Public Health England*, (September), 1–63.

Dhifi, W., Bellili, S., Jazi, S., Bahloul, N., and Mnif, W., 2016. Essential oils' chemical characterization and investigation of some biological activities: A critical review. *Medicines*, 3 (4), 25.

Di Domenico, E.G., Toma, L., Provot, C., Ascenzioni, F., Sperduti, I., Prignano, G., Gallo, M.T., Pimpinelli, F., Bordignon, V., Bernardi, T., and Ensoli, F., 2016. Development of an *in vitro* assay, based on the BioFilm Ring Test®, for rapid profiling of biofilm-growing bacteria. *Frontiers in Microbiology*, 7, 1429.

Donlan, R.M., 2002. Biofilms: Microbial life on surfaces. *Emerging Infectious Diseases*, 8 (9).

Doran, A.L., Morden, W.E., Dunn, K., and Edwards-Jones, V., 2009. Vapour-phase activities of essential oils against antibiotic sensitive and resistant bacteria including MRSA. *Letters in Applied Microbiology*, 48 (4), 387–392.

Dunne, W.M., 2002. Bacterial adhesion: seen any good biofilms lately? *Clinical microbiology reviews*, 15 (2), 155–66.

Eisenhut, M., 2007. The toxicity of essential oils. *International Journal of Infectious Diseases*, 11 (4), 365.

Elias, S. and Banin, E., 2012. Multi-species biofilms: Living with friendly neighbors. *FEMS Microbiology Reviews*, 36 (5), 990–1004.

Environmental Protection Agency, 2017. Methods and guidance for testing the efficacy of antimicrobials against biofilm bacteria on hard, non-porous surfaces [online]. *EPA*. Available from: https://www.epa.gov [Accessed 7 May 2018].

Eslahi, H., Fahimi, N., and Sardarian, A.R., 2017. Chemical composition of essential oils. *In*: S.M.B. Hashemi, A.M. Khaneghah, and A. de S. Sant'Ana, eds. *Essential Oils in Food Processing: Chemistry, Safety and Applications*. John Wiley & Sons, Ltd, 119–171.

EUCAST, 2018. European committee on antimicrobial susceptibility testing breakpoint tables for interpretation of MICs and zone diameters. *The European Committee on Antimicrobial Susceptibility Testing.*, (8), 0–77.

Evans, D.J., Allison, D.G., Brown, M.R.W., and Gilbert, P., 1991. Susceptibility of *Pseudomonas aeruginosa* and *Escherichia coli* biofilms towards ciprofloxacin: effect of specific growth rate. *Journal of Antimicrobial Chemotherapy*, 27 (2), 177–184.

Fajardo, A., Martínez-Martín, N., Mercadillo, M., Galán, J.C., Ghysels, B., Matthijs, S., Cornelis, P., Wiehlmann, L., Tümmler, B., Baquero, F., and Martínez, J.L., 2008. The neglected intrinsic resistome of bacterial pathogens. *PLoS ONE*, 3 (2), e1619.

Falcó, I., Verdeguer, M., Aznar, R., Sánchez, G., and Randazzo, W., 2019. Sanitizing food contact surfaces by the use of essential oils. *Innovative Food Science & Emerging Technologies*, 51, 220–228.

Faleiro, M.., 2011. The mode of antibacterial action of essential oils. *In*: A. Méndez-Vilas, ed. *Science against microbial pathogens: communicating current research and technological advances*. Badajoz: Formatex Research Center, 1143–1156.

Fazeli, H., Akbari, R., Moghim, S., Narimani, T., Arabestani, M.R., and Ghoddousi, A.R., 2012. Pseudomonas aeruginosa infections in patients, hospital means, and personnel's specimens. *Journal of Research in*

Medical Sciences, 17 (4), 332–7.

FDA, 2019. U.S. Code of Federal Regulations 21 Food and Drugs [online]. *Food and Drug Administration*. Available from: https://www.ecfr.gov/cgi-bin/text-idx?SID=3572f80f5791a3590d21dd4b14b3932d&mc=true&node=se21.3.182_120&rgn=div8 [Accessed 3 May 2019].

Feyaerts, A.F., Mathé, L., Luyten, W., De Graeve, S., Van Dyck, K., Broekx, L., and Van Dijck, P., 2018. Essential oils and their components are a class of antifungals with potent vapour-phase-mediated anti-*Candida* activity. *Scientific Reports*, 8 (1), 3958.

Fine, D.H., Markowitz, K., Furgang, D., Goldsmith, D., Ricci-Nittel, D., Charles, C.H., Peng, P., and Lynch, M.C., 2007. Effect of rinsing with an essential oil–containing mouthrinse on subgingival periodontopathogens. *Journal of Periodontology*, 78 (10), 1935–1942.

Firmino, D.F., Cavalcante, T.T.A., Gomes, G.A., Firmino, N.C.S., Rosa, L.D., De Carvalho, M.G., and Catunda, F.E.A., 2018. Antibacterial and antibiofilm activities of *Cinnamomum* Sp. essential oil and cinnamaldehyde: antimicrobial activities.

Fisher, K. and Phillips, C., 2009. The mechanism of action of a citrus oil blend against *Enterococcus faecium* and *Enterococcus faecalis*. *Journal of Applied Microbiology*, 106 (4), 1343–1349.

Flemming, H.-C., Neu, T.R., and Wozniak, D.J., 2007. The EPS matrix: the 'house of biofilm cells'. *Journal of Bacteriology*, 189 (22), 7945–7.

Friedman, M., Henika, P.R., Levin, C.E., and Mandrell, R.E., 2004. Antibacterial activities of plant essential oils and their components against *Escherichia coli* O157:H7 and *Salmonella enterica* in apple juice. *Journal of Agricultural and Food Chemistry*, 52 (19), 6042–6048.

Gebel, J., Exner, M., French, G., Chartier, Y., Christiansen, B., Gemein, S., Goroncy-Bermes, P., Hartemann, P., Heudorf, U., Kramer, A., Maillard,

J.-Y., Oltmanns, P., Rotter, M., and Sonntag, H.-G., 2013. The role of surface disinfection in infection prevention. *GMS Hygiene and Infection Control*, 8 (1), 1–12.

Gerdt, J.P. and Blackwell, H.E., 2014. Competition studies confirm two major barriers that can preclude the spread of resistance to quorum-sensing inhibitors in bacteria. *ACS Chemical Biology*, 9 (10), 2291–2299.

Ghabraie, M., Vu, K.D., Tata, L., Salmieri, S., and Lacroix, M., 2016. Antimicrobial effect of essential oils in combinations against five bacteria and their effect on sensorial quality of ground meat. *LWT - Food Science and Technology*, 66, 332–339.

Gill, A.O., Delaquis, P., Russo, P., and Holley, R.A., 2002. Evaluation of antilisterial action of cilantro oil on vacuum packed ham. *International Journal of Food Microbiology*, 73 (1), 83–92.

Goeres, D.M., Loetterle, L.R., Hamilton, M.A., Murga, R., Kirby, D.W., and Donlan, R.M., 2005. Statistical assessment of a laboratory method for growing biofilms. *Microbiology*, 151 (3), 757–762.

Goldbeck, J.C., Victoria, F.N., Motta, A., Savegnago, L., Jacob, R.G., Perin, G., Lenardão, E.J., and Padilha da Silva, W., 2014. Bioactivity and morphological changes of bacterial cells after exposure to 3-(p-chlorophenyl)thio citronellal. *LWT - Food Science and Technology*, 59 (2P1), 813–819.

Gómez-Estaca, J., López de Lacey, A., López-Caballero, M.E., Gómez-Guillén, M.C., and Montero, P., 2010. Biodegradable gelatin-chitosan films incorporated with essential oils as antimicrobial agents for fish preservation. *Food Microbiology*, 27 (7), 889–896.

Goñi, P., López, P., Sánchez, C., Gómez-Lus, R., Becerril, R., and Nerín, C., 2009. Antimicrobial activity in the vapour phase of a combination of cinnamon and clove essential oils. *Food Chemistry*, 116 (4), 982–989.

Gould, I. m. and Bal, A. m., 2013. New antibiotic agents in the pipeline and

how they can help overcome microbial resistance. *Virulence*, 4 (2), 185–191.

Govindasamy, R., Arumugam, S., and Simon, J.E., 2013. An assessment of the essential oil and aromatic plant industry with a focus on africa. *In*: *ACS Symposium Series*. 289–321.

Griffin, S.G., Markham, J.L., and Leach, D.N., 2000. An agar dilution method for the determination of the minimum inhibitory concentration of essential oils. *Journal of Essential Oil Research*, 12 (2), 249–255.

Guenther, E., 1948. The production of essential oils. *In*: E. Guenther, ed. *The Essential Oils Vol 1: History - Origin in Plants - Production - Analysis*. New York: van Nostrand Comapny, 85–218.

Guenther, E., 2013. *Essential Oils: History, Origin in Plants, Production and Analysis*. Read Books Ltd.

Gupta, P. and Deka, S., 2018. The menace of antimicrobial resistance. *Indian Journal of Community Health*, 30 (4), 317–322.

Guttenplan, S.B. and Kearns, D.B., 2013. Regulation of flagellar motility during biofilm formation. *FEMS Microbiology Reviews*, 37 (6), 849–871.

Gyawali, R. and Ibrahim, S.A., 2014. Natural products as antimicrobial agents. *Food Control*, 46 (2014), 412–429.

Hammer, K.A., Carson, C.F., Riley, T. V., and Nielsen, J.B., 2006. A review of the toxicity of *Melaleuca alternifolia* (tea tree) oil. *Food and Chemical Toxicology*, 44 (5), 616–625.

Hammer, K.A., Carson, C.F., and Riley, T. V, 1999. Antimicrobial activity of essential oils and other plant extracts. *Journal of Applied Microbiology*, 86 (6), 985–90.

Harbarth, S., Balkhy, H.H., Goossens, H., Jarlier, V., Kluytmans, J., Laxminarayan, R., Saam, M., Van Belkum, A., and Pittet, D., 2015. Antimicrobial resistance: One world, one fight! *Antimicrobial Resistance*

and Infection Control, 4 (1), 49.

Harmsen, M., Yang, L., Pamp, S.J., and Tolker-Nielsen, T., 2010. An update on *Pseudomonas aeruginosa* biofilm formation, tolerance, and dispersal. *FEMS Immunology and Medical Microbiology*, 59 (3), 253–268.

Hébert, C.D., Yuan, J., and Dieter, M.P., 1994. Comparison of the toxicity of cinnamaldehyde when administered by microencapsulation in feed or by corn oil gavage. *Food and Chemical Toxicology*, 32 (12), 1107–1115.

Helander, I.M. and Mattila-Sandholm, T., 2000. Fluorometric assessment of Gram-negative bacterial permeabilization. *Journal of Applied Microbiology*, 88 (2), 213–219.

Hems, R.S., Gulabivala, K., Ng, Y.-L., Ready, D., and Spratt, D.A., 2005. An *in vitro* evaluation of the ability of ozone to kill a strain of *Enterococcus faecalis*. *International Endodontic Journal*, 38 (1), 22–29.

Hengge, R., 2014. The general stress response in Gram-negative bacteria. *In*: G. Storz and R. Hengge, eds. *Bacterial Stress Responses*. American Society of Microbiology, 251–289.

Hense, B.A. and Schuster, M., 2015. Core Principles of Bacterial Autoinducer Systems. *Microbiology and Molecular Biology Reviews*, 79 (1), 153–169.

HM Government, 2019. Tackling antimicrobial resistance 2019–2024: The UK's five-year national action plan [online]. Available from: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/784894/UK_AMR_5_year_national_action_plan.pdf.

Høiby, N., Ciofu, O., and Bjarnsholt, T., 2010. *Pseudomonas aeruginosa* biofilms in cystic fibrosis. *Future Microbiology*, 5 (11), 1663–1674.

Holah, J.T. and Thorpe, R.H., 1990. Cleanability in relation to bacterial retention on unused and abraded domestic sink materials. *Journal of Applied Bacteriology*, 69 (4), 599–608.

Hoskovec, M., Grygarová, D., Cvačka, J., Streinz, L., Zima, J., Verevkin, S.P., and Koutek, B., 2005. Determining the vapour pressures of plant volatiles from gas chromatographic retention data. *Journal of Chromatography A*, 1083 (1–2), 161–172.

Houdkova, M., Urbanova, K., Doskocil, I., Rondevaldova, J., Novy, P., Nguon, S., Chrun, R., and Kokoska, L., 2018. In vitro growth-inhibitory effect of Cambodian essential oils against pneumonia causing bacteria in liquid and vapour phase and their toxicity to lung fibroblasts. *South African Journal of Botany*, 118, 85–97.

Howe, R.A. and Andrews, J.M., 2012. BSAC standardized disc susceptibility testing method (version 11). *Journal of Antimicrobial Chemotherapy*, 67 (12), 2783–2784.

Inouye, S., Takizawa, T., and Yamaguchi, H., 2001. Antibacterial activity of essential oils and their major constituents against respiratory tract pathogens by gaseous contact. *The Journal of antimicrobial chemotherapy*, 47 (5), 565–573.

Janssen, A.M., Chin, N.L.J., Scheffer, J.J.C., and Svendsen, A.B., 1986. Screening for antimicrobial activity of some essential oils by the agar overlay technique - Statistics and correlations. *Pharmaceutisch Weekblad Scientific Edition*, 8 (6), 289–292.

Jayawardena, B. and Smith, R.M., 2010. Superheated water extraction of essential oils from *cinnamomum zeylanicum* (L.). *Phytochemical Analysis*, 21 (5), 470–472.

Jenner, P.M., Hagan, E.C., Taylor, J.M., Cook, E.L., and Fitzhugh, O.G., 1964. Food flavourings and compounds of related structure I. Acute oral toxicity. *Food and Cosmetics Toxicology*, 2, 327–343.

Ji, H., Kim, H., Beuchat, L.R., and Ryu, J.-H.H., 2019. Synergistic antimicrobial activities of essential oil vapours against *Penicillium corylophilum* on a laboratory medium and beef jerky. *International Journal of Food*

Microbiology, 291, 104–110.

Jiao, Y., Cody, G.D., Harding, A.K., Wilmes, P., Schrenk, M., Wheeler, K.E., Banfield, J.F., and Thelen, M.P., 2010. Characterization of extracellular polymeric substances from acidophilic microbial biofilms. *Applied and Environmental Microbiology*, 76 (9), 2916–2922.

Jorgensen, J.H. and Ferraro, M.J., 2009. Antimicrobial susceptibility testing: a review of general principles and contemporary practices. *Medical Microbiology*, 49 (11), 1749–55.

Ju, J., Xu, X., Xie, Y., Guo, Y., Cheng, Y., Qian, H., and Yao, W., 2018. Inhibitory effects of cinnamon and clove essential oils on mold growth on baked foods. *Food Chemistry*, 240 (July 2017), 850–855.

Kačániová, M., Terentjeva, M., Vukovic, N., Puchalski, C., Roychoudhury, S., Kunová, S., Klūga, A., Tokár, M., Kluz, M., and Ivanišová, E., 2017. The antioxidant and antimicrobial activity of essential oils against *Pseudomonas* spp. isolated from fish. *Saudi Pharmaceutical Journal*, 25 (8), 1108–1116.

Kalia, M., Yadav, V.K., Singh, P.K., Sharma, D., Pandey, H., Narvi, S.S., and Agarwal, V., 2015. Effect of cinnamon oil on quorum sensing-controlled virulence factors and biofilm formation in *Pseudomonas aeruginosa*. *PLoS ONE*, 10 (8).

Kaplan, J.B., 2010. Biofilm dispersal: Mechanisms, clinical implications, and potential therapeutic uses. *Journal of Dental Research*, 89 (3), 205–218.

Kavanaugh, N.L. and Ribbeck, K., 2012. Selected antimicrobial essential oils eradicate *Pseudomonas* spp. and *Staphylococcus aureus* biofilms. *Applied and Environmental Microbiology*, 78 (11), 4057–4061.

Kazemi, M. and Mokhtariniya, S., 2016. Essential oil composition of bark of Cinnamomum zeylanicum. *Journal of Essential Oil Bearing Plants*, 19 (3), 786–789.

Kerr, K.G. and Snelling, A.M., 2009. *Pseudomonas aeruginosa*: a formidable and ever-present adversary. *Journal of Hospital Infection*, 73, 338–334.

KH, S., AA, A.I., TO, O., RA, R., and AJ, O., 2017. Bacterial contamination of hospital surfaces according to material make, last time of contact and last time of cleaning/disinfection. *Journal of Bacteriology & Parasitology*, 08 (03).

Kim, Y.G., Lee, J.H., Kim, S. Il, Baek, K.H., and Lee, J., 2015. Cinnamon bark oil and its components inhibit biofilm formation and toxin production. *International Journal of Food Microbiology*, 195, 30–39.

Kloucek, P., Smid, J., Frankova, A., Kokoska, L., Valterova, I., and Pavela, R., 2012. Fast screening method for assessment of antimicrobial activity of essential oils in vapor phase. *Food Research International*, 47 (2), 161–165.

Kommerein, N., Doll, K., Stumpp, N.S., and Stiesch, M., 2018. Development and characterization of an oral multispecies biofilm implant flow chamber model. *PLoS ONE*, 13 (5), 1–15.

Kortright, K.E., Chan, B.K., Koff, J.L., and Turner, P.E., 2019. Phage therapy: A renewed approach to combat antibiotic-resistant bacteria. *Cell Host & Microbe*, 25 (2), 219–232.

Křůmal, K., Kubátková, N., Večeřa, Z., and Mikuška, P., 2015. Antimicrobial properties and chemical composition of liquid and gaseous phases of essential oils. *Chemical Papers*, 69 (8), 1084–1092.

Kuhn, D., Ziem, R., Scheibel, T., Buhl, B., Vettorello, G., Pacheco, L.A., Heidrich, D., Kauffmann, C., de Freitas, E.M., Ethur, E.M., and Hoehne, L., 2019. Antibiofilm activity of the essential oil of *Campomanesia aurea* O. Berg against microorganisms causing food borne diseases. *LWT*, 108, 247–252.

Lacko, M., Wang, N., Sovová, K., Pásztor, P., and Španěl, P., 2019. Addition of a fast GC to SIFT-MS for analyses of individual monoterpenes in

mixtures. *Atmospheric Measurement Techniques Discussions*, 1–23.

Laird, K., Armitage, D., and Phillips, C., 2012. Reduction of surface contamination and biofilms of *Enterococcus* sp. and *Staphylococcus aureus* using a citrus-based vapour. *Journal of Hospital Infection*, 80 (1), 61–66.

Lakshminarayanan, R., Ye, E., Young, D.J., Li, Z., and Loh, X.J., 2018. Recent advances in the development of antimicrobial nanoparticles for combating resistant pathogens. *Advanced Healthcare Materials*, 7 (13).

Lalancette, C., Charron, D., Laferrière, C., Dolcé, P., Déziel, E., Prévost, M., and Bédard, E., 2017. Hospital drains as reservoirs of *Pseudomonas aeruginosa*: multiple-locus variable-number of tandem repeats analysis genotypes recovered from faucets, sink surfaces and patients. *Pathogens*, 6 (3), 36.

Langeveld, W.T., Veldhuizen, E.J.A., and Burt, S.A., 2014. Synergy between essential oil components and antibiotics: A review. *Critical Reviews in Microbiology*, 40 (1), 76–94.

Ledwoch, K., Said, J., Norville, P., and Maillard, J. -Y., 2019. Artificial dry surface biofilm models for testing the efficacy of cleaning and disinfection. *Letters in Applied Microbiology*, 68 (4), 329–336.

Lee, G., Kim, Y., Kim, H., Beuchat, L.R., and Ryu, J.-H.H., 2018. Antimicrobial activities of gaseous essential oils against Listeria monocytogenes on a laboratory medium and radish sprouts. *International Journal of Food Microbiology*, 265 (October 2017), 49–54.

Lee, M.S., Choi, J., Posadzki, P., and Ernst, E., 2012. Aromatherapy for health care: An overview of systematic reviews. *Maturitas*.

Leggio, A., Leotta, V., Belsito, E.L., Di Gioia, M.L., Romio, E., Santoro, I., Taverna, D., Sindona, G., and Liguori, A., 2017. Aromatherapy: Composition of the gaseous phase at equilibrium with liquid bergamot essential oil. *Chemistry Central Journal*, 11 (1), 111.

Levy, S.B. and Bonnie, M., 2004. Antibacterial resistance worldwide: Causes, challenges and responses. *Nature Medicine*, 10 (12S), S122–S129.

Li, J., Perdue, E.M., Pavlostathis, S.G., and Araujo, R., 1998. Physicochemical properties of selected monoterpenes. *Environment International*, 24 (3), 353–358.

Li, X.Z., Zhang, L., and Poole, K., 1998. Role of the multidrug efflux systems of *Pseudomonas aeruginosa* in organic solvent tolerance. *Journal of Bacteriology*, 180 (11), 2987–2991.

Lin, P.-C., Lee, P.-H., Tseng, S.-J., Lin, Y.-M., Chen, S.-R., and Hou, W.-H., 2019. Effects of aromatherapy on sleep quality: A systematic review and meta-analysis. *Complementary Therapies in Medicine*, 45, 156–166.

Lobanovska, M. and Pilla, G., 2017. Penicillin's discovery and antibiotic resistance: Lessons for the future? *Yale Journal of Biology and Medicine*, 90 (1), 135–145.

Lodhia, M.H., Bhatt, K.R., and Thaker, V.S., 2009. Antibacterial activity of essential oils from palmarosa, evening primrose, lavender and tuberose. *Indian Journal of Pharmaceutical Sciences*, 71 (2), 134–6.

Lopez-Romero, J.C., González-Ríos, H., Borges, A., and Simões, M., 2015. Antibacterial effects and mode of action of selected essential oils components against *Escherichia coli* and *Staphylococcus aureus*. *Evidence-based complementary and alternative medicine : eCAM*, 2015, 795435.

López, E.M., Vargas, G.P.V., Palou, E., and Malo, A.L., 2018. *Penicillium expansum* inhibition on bread by lemongrass essential Oil in vapor phase. *Journal of Food Protection*, 81 (3), 467–471.

López, P., Sánchez, C., Batlle, R., and Nerín, C., 2007. Vapor-phase activities of cinnamon, thyme, and oregano essential oils and key constituents against foodborne microorganisms. *Journal of Agricultural and Food Chemistry*, 55 (11), 4348–4356.

Lu, W.C., Huang, D.W., Wang, C.C.R., Yeh, C.H., Tsai, J.C., Huang, Y.T., and Li, P.H., 2018. Preparation, characterization, and antimicrobial activity of nanoemulsions incorporating citral essential oil. *Journal of Food and Drug Analysis*, 26 (1), 82–89.

Lüdecke, C., Jandt, K.D., Siegismund, D., Kujau, M.J., Zang, E., Rettenmayr, M., Bossert, J., and Roth, M., 2014. Reproducible biofilm cultivation of chemostat-grown Escherichia coli and investigation of bacterial adhesion on biomaterials using a non-constant-depth film fermenter. *PLoS ONE*, 9 (1).

Macià, M.D., Rojo-Molinero, E., and Oliver, A., 2014. Antimicrobial susceptibility testing in biofilm-growing bacteria. *Clinical Microbiology and Infection*, 20 (10), 981–990.

Magi, G., Marini, E., and Facinelli, B., 2015. Antimicrobial activity of essential oils and carvacrol, and synergy of carvacrol and erythromycin, against clinical, erythromycin-resistant Group A Streptococci. *Frontiers in Microbiology*, 6 (MAR), 1–7.

Magiorakos, A.P., Srinivasan, A., Carey, R.B., Carmeli, Y., Falagas, M.E., Giske, C.G., Harbarth, S., Hindler, J.F., Kahlmeter, G., Olsson-Liljequist, B., Paterson, D.L., Rice, L.B., Stelling, J., Struelens, M.J., Vatopoulos, A., Weber, J.T., and Monnet, D.L., 2012. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: An international expert proposal for interim standard definitions for acquired resistance. *Clinical Microbiology and Infection*, 18 (3), 268–281.

Mah, T.-F.F.C.F.C. and O'Toole, G.A., 2001. Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology*, 9 (1), 34–39.

Maisanaba, S., Llana-Ruiz-Cabello, M., Gutiérrez-Praena, D., Pichardo, S., Puerto, M., Prieto, A.I., Jos, A., and Cameán, A.M., 2017. New advances in active packaging incorporated with essential oils or their main components for food preservation. *Food Reviews International*, 33 (5), 447–515.

Malic, S., Emanuel, C., Lewis, M.A., and Williams, D.W., 2013. Antimicrobial activity of novel mouthrinses against planktonic cells and biofilms of pathogenic microorganisms. *Microbiology Discovery*, 1 (1), 11.

Mallavarapu, G.R., Ramesh, S., Chandrasekhara, R.S., Rajeswara Rao, B.R., Kaul, P.N., and Bhattacharya, A.K., 1995. Investigation of the essential oil of cinnamon leaf grown at Bangalore and Hyderabad. *Flavour and Fragrance Journal*, 10 (4), 239–242.

Man, A., Santacroce, L., Jacob, R., Mare, A., and Man, L., 2019. Antimicrobial activity of six essential oils against a group of human pathogens: A comparative study. *Pathogens*, 8 (1), 15.

Mann, C.M. and Markham, J.L., 1998. A new method for determining the minimum inhibitory concentration of essential oils. *Journal of Applied Microbiology*, 84 (4), 538–544.

Manso, S., Pezo, D., Gómez-Lus, R., and Nerín, C., 2014. Diminution of aflatoxin B1 production caused by an active packaging containing cinnamon essential oil. *Food Control*, 45, 101–108.

Marino, M., Maifreni, M., Baggio, A., and Innocente, N., 2018. Inactivation of foodborne bacteria biofilms by aqueous and gaseous ozone. *Frontiers in Microbiology*, 9 (AUG), 1–12.

Markarian, S.A., Poladyan, A.A., Kirakosyan, G.R., Trchounian, A.A., and Bagramyan, K.A., 2002. Effect of diethylsulphoxide on growth, survival and ion exchange of Escherichia coli. *Letters in Applied Microbiology*, 34 (6), 417–421.

Martens, E. and Demain, A.L., 2017. An overview of the industrial aspects of antibiotic discovery. *In*: *Microbial Resources: From Functional Existence in Nature to Applications*. Academic Press, 149–168.

Maruzzella, J.C. and Sicurella, N.A., 1960. Antibacterial activity of essential oil vapors. *Journal of the American Pharmaceutical Association*, 49 (11), 692–694.

May, J., Chan, C.H., King, A., Williams, L., and French, G.L., 2000. Time-kill studies of tea tree oils on clinical isolates. *The Journal of Antimicrobial Chemotherapy*, 45 (5), 639–643.

Mayaud, L., Carricajo, A., Zhiri, A., and Aubert, G., 2008. Comparison of bacteriostatic and bactericidal activity of 13 essential oils against strains with varying sensitivity to antibiotics. *Letters in Applied Microbiology*, 47 (3), 167–173.

Mayor, S., 2019. Doctors to get real time data to support antibiotic prescribing and reduce resistance. *BMJ (Clinical research ed.)*, 364, 406.

Mekonnen, A., Tesfaye, S., Christos, S.G., Dires, K., Zenebe, T., Zegeye, N., Shiferaw, Y., and Lulekal, E., 2019. Evaluation of skin irritation and acute and subacute oral toxicity of *Lavandula angustifolia* essential oils in rabbit and mice. *Journal of Toxicology*, 2019, 1–8.

Van Merode, A.E.J., Van Der Mei, H.C., Busscher, H.J., and Krom, B.P., 2006. Influence of culture heterogeneity in cell surface charge on adhesion and biofilm formation by Enterococcus faecalis. *Journal of Bacteriology*, 188 (7), 2421–2426.

Merritt, J.H., Kadouri, D.E., and O'Toole, G.A., 2011. Growing and analyzing static biofilms. *Current Protocols in Microbiology*, (SUPPL. 22), 1–18.

Messager, S., Hammer, K.A., Carson, C.F., and Riley, T. V., 2005. Assessment of the antibacterial activity of tea tree oil using the European EN 1276 and EN 12054 standard suspension tests. *Journal of Hospital Infection*, 59 (2), 113–125.

Michael, C.A., Dominey-Howes, D., Labbate, M., Maria, C., Elisabeth, J., Pitout, J., and Walsh, F., 2014. The antimicrobial resistance crisis: causes, consequences, and management. *Frontiers in Public Health*, 2 (145), 1–8.

Miksusanti, M., Sri Laksmi Jenie, B., Pontjo Priosoeryanto, B., Syarief, R., and Gatot, T.R., 2008. Mode of action Temu Kunci (*Kaempferia pandurata*)

essential oil on *E. coli* K1.1 cell determined by leakage of material cell and salt tolerance assays. *HAYATI Journal of Biosciences*, 15 (2), 56–60.

Miller, S.I., 2016. Antibiotic resistance and regulation of the Gram-negative bacterial outer membrane barrier by host innate immune molecules. *mBio*, 7 (5), 1541–16.

Millezi, A.F., Piccoli, R.H., Oliveira, J.M., and Pereira, M.O., 2016. Anti-biofim and antibacterial effect of essential oils and their major compounds. *Journal of Essential Oil Bearing Plants*, 19 (3), 624–631.

Mittal, R.P., Rana, A., and Jaitak, V., 2019. Essential oils: An impending substitute of synthetic antimicrobial agents to overcome antimicrobial resistance. *Current Drug Targets*, 20 (6), 605–624.

Moat, J., Cargill, J., Shone, J., and Upton, M., 2009. Application of a novel decontamination process using gaseous ozone. *Canadian Journal of Microbiology*, 55 (8), 928–933.

Moon, S.E., Kim, H.Y., and Cha, J.D., 2011. Synergistic effect between clove oil and its major compounds and antibiotics against oral bacteria. *Archives of Oral Biology*, 56 (9), 907–916.

Mosselhy, D.A., He, W., Hynönen, U., Meng, Y., Mohammadi, P., Palva, A., Feng, Q., Hannula, S.P., Nordström, K., and Linder, M.B., 2018. Silica–gentamicin nanohybrids: Combating antibiotic resistance, bacterial biofilms, and in vivo toxicity. *International Journal of Nanomedicine*, 13, 7939–7957.

Mourey, A. and Canillac, N., 2002. Anti-*Listeria monocytogenes* activity of essential oils components of conifers. *Food Control*, 13 (4–5), 289–292.

Mulani, M.S., Kamble, E.E., Kumkar, S.N., Tawre, M.S., and Pardesi, K.R., 2019. Emerging strategies to combat ESKAPE pathogens in the era of antimicrobial resistance: A review. *Frontiers in Microbiology*, 10, 539.

Muniesa, A., Escobar-Dodero, J., Silva, N., Henríquez, P., Bustos, P., Perez,

A.M., and Mardones, F.O., 2019. Effectiveness of disinfectant treatments for inactivating *Piscirickettsia salmonis*. *Preventive Veterinary Medicine*, 167, 196–201.

Munita, J.M. and Arias, C.A., 2016. Mechanisms of antibiotic resistance. *In*: I. Kudva, N. Cornick, P. Plummer, Q. Zhang, T. Nicholson, J. Bannantine, and B. Bellaire, eds. *Virulence Mechanisms of Bacterial Pathogens*.

Murtey, M. Das and Ramasamy, P., 2016. Sample preparations for scanning electron microscopy – Life sciences. *In*: M. Janecek, ed. *Modern Electron Microscopy in Physical and Life Sciences*. InTech, 161–185.

Muzzarelli, L., Force, M., and Sebold, M., 2006. Aromatherapy and reducing preprocedural anxiety: A controlled prospective study. *Gastroenterology Nursing*, 29 (6), 466–471.

Nabavi, S.F., Di Lorenzo, A., Izadi, M., Sobarzo-Sánchez, E., Daglia, M., and Nabavi, S.M., 2015. Antibacterial effects of cinnamon: from farm to food, cosmetic and pharmaceutical industries. *Nutrients*, 7 (9), 7729–48.

Nafis, A., Kasrati, A., Jamali, C.A., Mezrioui, N., Setzer, W., Abbad, A., and Hassani, L., 2019. Antioxidant activity and evidence for synergism of *Cannabis sativa* (L.)essential oil with antimicrobial standards. *Industrial Crops and Products*, 137, 396–400.

Narayanasamy, K., Elangovan, E., Keerthi, D., Jagadeeswari, S., Krithiga, B., Padmanabhan, V., and Periyasamy, S., 2019. Antimicrobial activity of selected essential oils against antibiotic resistant organisms. *Asian Journal of Pharmacy and Pharmacology*, 5 (3), 503–512.

Nath, S.S., Pandey, C., and Roy, D., 2012. A near fatal case of high dose peppermint oil ingestion- Lessons learnt. *Indian Journal of Anaesthesia*, 56 (6), 582–584.

Nazzaro, F., Fratianni, F., Coppola, R., and De Feo, V., 2017. Essential oils and antifungal activity. *Pharmaceuticals*, 10 (4), 1–20.

Nazzaro, F., Fratianni, F., D'Acierno, A., Coppola, R., Jesus Ayala-Zavala, F., Gomez da Cruz, A., and De Feo, V., 2019. Essential oils and microbial communication. *In*: H. El-Shemy, ed. *Essential Oils - Oils of Nature*. IntechOpen.

Nazzaro, F., Fratianni, F., De Martino, L., Coppola, R., and De Feo, V., 2013. Effect of essential oils on pathogenic bacteria. *Pharmaceuticals*, 6 (12), 1451–1474.

Nedorostova, L., Kloucek, P., Kokoska, L., Stolcova, M., and Pulkrabek, J., 2009. Antimicrobial properties of selected essential oils in vapour phase against foodborne bacteria. *Food Control*, 20 (2), 157–160.

Niederman, M.S., 2005. Principles of appropriate antibiotic use. *International Journal of Antimicrobial Agents*, 26 (3), 170–175.

Nikaido, H., 2009. Multidrug Resistance in Bacteria. *Annual Review of Biochemistry*, 78 (1), 119–146.

Nostro, A., Marino, A., Blanco, A.R., Cellini, L., Di Giulio, M., Pizzimenti, F., Roccaro, A.S., and Bisignano, G., 2009. *In vitro* activity of carvacrol against staphylococcal preformed biofilm by liquid and vapour contact. *Journal of Medical Microbiology*, 58 (6), 791–797.

O'Neill, J., 2014. Review on antimicrobial resistance [online]. Available from: https://amr-review.org/sites/default/files/AMR Review Paper - Tackling a crisis for the health and wealth of nations_1.pdf.

O'Neill, J., 2016. Tackling drug-resistant infections globally: Final report and recommendations [online]. Available from: https://amr-review.org/sites/default/files/160518_Final paper_with cover.pdf.

O'Toole, G., Kaplan, H.B., and Kolter, R., 2000. Biofilm formation as microbial development. *Annual Review of Microbiology*, 54 (1), 49–79.

O'Toole, G.A., 2003. To Build a Biofilm. *Journal of Bacteriology*, 185 (9), 2687–2689.

Obama White House, 2015. President's 2016 budget proposes historic investment to combat antibiotic-resistant bacteria to protect public health [online]. Available from: https://obamawhitehouse.archives.gov/the-press-office/2015/01/27/fact-sheet-president-s-2016-budget-proposes-historic-investment-combat-a [Accessed 9 Jun 2019].

Ojagh, S.M., Rezaei, M., Seyed, &, Razavi, H., and Razavi, S.H., 2014. Improvement of the storage quality of frozen rainbow trout by chitosan coating incorporated with cinnamon oil. *Journal of Aquatic Food Product Technology*, 23 (2), 146–154.

Oliveira, F. de S., Freitas, T.S. de, Cruz, R.P. da, Costa, M. do S., Pereira, R.L.S., Quintans-Júnior, L.J., Andrade, T. de A., Menezes, P. dos P., Sousa, B.M.H. de, Nunes, P.S., Serafini, M.R., Menezes, I.R.A. de, Araújo, A.A. de S., and Coutinho, H.D.M., 2017. Evaluation of the antibacterial and modulatory potential of α-bisabolol, β-cyclodextrin and α-bisabolol/β-cyclodextrin complex. *Biomedicine and Pharmacotherapy*, 92, 1111–1118.

de Oliveira, P.F., Alves, J.M., Damasceno, J.L., Oliveira, R.A.M., Júnior Dias, H., Crotti, A.E.M., and Tavares, D.C., 2015. Cytotoxicity screening of essential oils in cancer cell lines. *Brazilian Journal of Pharmacognosy*, 25 (2), 183–188.

Otter, J.A., Yezli, S., Schouten, M.A., Van Zanten, A.R.H., Houmes-Zielman, G., and Nohlmans-Paulssen, M.K., 2010. Hydrogen peroxide vapor decontamination of an intensive care unit to remove environmental reservoirs of multidrug-resistant gram-negative rods during an outbreak. *American Journal of Infection Control*, 38 (9), 754–756.

Palmer, A.C. and Kishony, R., 2014. Opposing effects of target overexpression reveal drug mechanisms. *Nature Communications*, 5, 1–8.

Palmer, J., Flint, S., and Brooks, J., 2007. Bacterial cell attachment, the beginning of a biofilm. *Journal of Industrial Microbiology and Biotechnology*, 34 (9), 577–588.

Pantanella, F., Valenti, P., Natalizi, T., Passeri, D., and Berlutti, F., 2013. Analytical techniques to study microbial biofilm on abiotic surfaces: pros and cons of the main techniques currently in use. *Annali de Igiene*, 25 (1), 31–42.

Paranagama, P.A., Wimalasena, S., Jayatilake, G.S., Jayawardena, A.L., Senanayake, U.M., and Mubarak, A.M., 2001. A comparison of essential oil constituents of bark, leaf, root and fruit of cinnamon (*cinnamomum zeylanicum* blume) grown in Sri Lanka. *Journal of the National Science Foundation of Sri Lanka*, 29 (3–4), 147–153.

Park, J.B., Kang, J.H., and Song, K. Bin, 2018. Antibacterial activities of a cinnamon essential oil with cetylpyridinium chloride emulsion against *Escherichia coli* O157:H7 and *Salmonella* Typhimurium in basil leaves. *Food Science and Biotechnology*, 27 (1), 47–55.

Pavithra, D. and Doble, M., 2008. Biofilm formation, bacterial adhesion and host response on polymeric implants - Issues and prevention. *Biomedical Materials*, 3 (3), SMI.

Pei, R., Zhou, F., Ji, B., and Xu, J., 2009. Evaluation of Combined Antibacterial Effects of Eugenol, Cinnamaldehyde, Thymol, and Carvacrol against *E. coli* with an Improved Method. *Journal of Food Science*, 74 (7), M379–M383.

Pericolini, E., Colombari, B., Ferretti, G., Iseppi, R., Ardizzoni, A., Girardis, M., Sala, A., Peppoloni, S., and Blasi, E., 2018. Real-time monitoring of *Pseudomonas aeruginosa* biofilm formation on endotracheal tubes in vitro. *BMC Microbiology*, 18 (1), 84.

Perry, R.H., Perry, S., Green, D.W., and Maloney, J.O., 1997. *Gasification, in: R. H. Perry, (Ed.), Perry's Chemical Engineers' Handbook, 7th ed.* McGraw-Hill New York.

Peterson, E. and Kaur, P., 2018. Antibiotic resistance mechanisms in bacteria: Relationships between resistance determinants of antibiotic producers,

environmental bacteria, and clinical pathogens. *Frontiers in Microbiology*, 9 (NOV), 1–21.

Petrachi, T., Resca, E., Piccinno, M.S., Biagi, F., Strusi, V., Dominici, M., and Veronesi, E., 2017. An alternative approach to investigate biofilm in medical devices: a feasibility study. *International Journal of Environmental Research and Public Health*, 14 (12).

Petrova, O.E., Sauer, K., Opin, C., and Author, M., 2016. Escaping the biofilm in more than one way: Desorption, detachment or dispersion. *Current Opinion in Microbiology*, 30, 67–78.

Pope, D.C. and Oliver, W.T., 1966. Dimethyl sulfoxide (DMSO). *Canadian Journal of Comparative Medicine and Veterinary Science*, 30 (1), 3–8.

Porter, N.G. and Wilkins, A.L., 1998. Chemical, physical and antimicrobial properties of essential oils of *Leptospermum scoparium* and *Kunzea ericoides*. *Phytochemistry*, 50 (3), 407–415.

Prabuseenivasan, S., Jayakumar, M., and Ignacimuthu, S., 2006. In vitro antibacterial activity of some plant essential oils. *BMC complementary and alternative medicine*, 6, 39.

Presterl, E., Diab-El Schahawi, M., Lusignani, L.S., Paula, H., and Reilly, J.S., 2019. Reprocessing: Cleansing, Disinfection, Sterilization. *In*: E. Presterl, M. Diab-El Schahawi, and J.S. Reilly, eds. *Basic Microbiology and Infection Control for Midwives*. Cham: Springer International Publishing, 35–49.

Prestinaci, F., Pezzotti, P., and Pantosti, A., 2015. Antimicrobial resistance: a global multifaceted phenomenon. *Pathogens and Global Health*, 109 (7), 309–318.

Priti, V. and Shridhar, P., 2012. Effect of essential oils on MDR pathogens: a comparative study. *Journal of Environmental Research and Development*, 6 (3), 462–466.

Public Health England, 2018. Research reveals levels of inappropriate prescriptions in England [online]. *PHE 2018*. Available from: https://www.gov.uk/government/news/research-reveals-levels-of-inappropriate-prescriptions-in-england [Accessed 10 Jun 2019].

Puškárová, A., Bučková, M., Kraková, L., Pangallo, D., and Kozics, K., 2017. The antibacterial and antifungal activity of six essential oils and their cyto/genotoxicity to human HEL 12469 cells. *Scientific Reports*, 7 (1), 1–11.

Quick, J., Cumley, N., Wearn, C.M., Niebel, M., Constantinidou, C., Thomas, C.M., Pallen, M.J., Moiemen, N.S., Bamford, A., Oppenheim, B., and Loman, N.J., 2014. Seeking the source of *Pseudomonas aeruginosa* infections in a recently opened hospital: an observational study using whole-genome sequencing. *BMJ Open*, 4 (e006278), 1–10.

Raina, V.K., Srivastava, S.K., Aggarwal, K.K., Ramesh, S., and Kumar, S., 2001. Essential oil composition of *Cinnamomum zeylanicum* Blume leaves from Little Andaman, India. *Flavour and Fragrance Journal*, 16 (5), 374–376.

Ranasinghe, P., Jayawardana, R., Galappaththy, P., Constantine, G.R., de Vas Gunawardana, N., and Katulanda, P., 2012. Efficacy and safety of ‘true’ cinnamon (*Cinnamomum zeylanicum*) as a pharmaceutical agent in diabetes: a systematic review and meta-analysis. *Diabetic Medicine*, 29 (12), 1480–1492.

Rather, M.A., Dar, B.A., Sofi, S.N., Bhat, B.A., and Qurishi, M.A., 2016. *Foeniculum vulgare*: A comprehensive review of its traditional use, phytochemistry, pharmacology, and safety. *Arabian Journal of Chemistry*, 9, S1574–S1583.

Reis, D. and Jones, T., 2017. Aromatherapy: Using essential oils as a supportive therapy. *Clinical Journal of Oncology Nursing*, 21 (1), 16–19.

Reisner, A., Haagensen, J.A.J., Schembri, M.A., Zechner, E.L., and Molin, S.,

2003. Development and maturation of *Escherichia coli* K-12 biofilms. *Molecular microbiology*, 48 (4), 933–46.

Remmal, A., Bouchikhi, T., Tantaoui-Elaraki, A., Ettayebi, M., Tantaoui-Eraraki, A., and Ettayebi, M., 1993. Inhibition of antibacterial activity of essential oils by tween 80 and ethanol in liquid medium. *Journal de Pharmacie de Belgique*, 48 (5), 352–6.

Reyes-Jurado, F., Navarro-Cruz, A.R., Ochoa-Velasco, C.E., Palou, E., López-Malo, A., and Ávila-Sosa, R., 2019. Essential oils in vapor phase as alternative antimicrobials: A review. *Critical Reviews in Food Science and Nutrition*, 1–10.

Riddick, J.A., Bunger, W.B., and Sakano, T.K., 1986. *Organic solvents: physical properties and methods of purification*. 4th ed. New York: John Wiley and Sons.

Rodríguez, A., Batlle, R., and Nerín, C., 2007. The use of natural essential oils as antimicrobial solutions in paper packaging. Part II. *Progress in Organic Coatings*, 60 (1), 33–38.

Rogers, G.B., Carroll, M.P., and Bruce, K.D., 2012. Enhancing the utility of existing antibiotics by targeting bacterial behaviour? *British Journal of Pharmacology*, 165 (4), 845–857.

Romulo, A., Zuhud, E.A.M., Rondevaldova, J., and Kokoska, L., 2018. Screening of in vitro antimicrobial activity of plants used in traditional Indonesian medicine. *Pharmaceutical Biology*, 56 (1), 287–293.

Van Roon, A., Parsons, J.R., Te Kloeze, A.M., and Govers, H.A.J., 2005. Fate and transport of monoterpenes through soils. Part I. Prediction of temperature dependent soil fate model input-parameters. *Chemosphere*, 61 (5), 599–609.

Rosenblatt-Farrell, N., 2009. The landscape of antibiotic resistance. *Environmental Health Perspectives*, 117 (6), A244-50.

Roshan, N., Hammer, K.A., and Riley, T. V., 2018. Non-conventional antimicrobial and alternative therapies for the treatment of *Clostridium difficile* infection. *Anaerobe*, 49, 103–111.

Sabaeifard, P., Abdi-Ali, A., Soudi, M.R., and Dinarvand, R., 2014. Optimization of tetrazolium salt assay for *Pseudomonas aeruginosa* biofilm using microtiter plate method. *Journal of Microbiological Methods*, 105, 134–140.

Sakkas, H., Gousia, P., Economou, V., Sakkas, V., Petsios, S., and Papadopoulou, C., 2016. In vitro antimicrobial activity of five essential oils on multi-drug resistant Gram-negative clinical isolates. *Journal of Intercultural Ethnopharmacology*, 5 (3), 212.

Sambasivarao, D., Weiner, J.H., Kroger, A., and Goethe, J.W., 1991. Dimethyl Sulfoxide Reductase of Escherichia coli: an investigation of function and assembly by use of in vivo complementation. *Journal of Bacteriology*, 173 (19), 5935–5943.

Sandle, T., 2017. The European approach to disinfectant qualification. *La Vague*, 52 (January), 45–48.

Sanla-Ead, N., Jangchud, A., Chonhenchob, V., and Suppakul, P., 2011. Antimicrobial activity of cinnamaldehyde and eugenol and their activity after incorporation into cellulose-based packaging films. *Packaging Technology and Science*, 25 (1), 7–17.

Santajit, S. and Indrawattana, N., 2016. Mechanisms of antimicrobial resistance in ESKAPE pathogens. *BioMed Research International*, 2016, 1–8.

Sarkic, A. and Stappen, I., 2018. Essential oils and their single compounds in cosmetics—A critical review. *Cosmetics*, 5 (1), 11.

Saviuc, C.-M., Drumea, V., Olariu, L., Chifiriuc, M.-C., Bezirtzoglou, E., and Lazăr, V., 2015. Essential oils with microbicidal and antibiofilm activity. *Current Pharmaceutical Biotechnology*, 16 (2), 137–51.

Schmidt, M.G., Attaway, H.H., Sharpe, P.A., John, J., Sepkowitz, K.A., Morgan, A., Fairey, S.E., Singh, S., Steed, L.L., Cantey, J.R., Freeman, K.D., Michels, H.T., and Salgado, C.D., 2012. Sustained reduction of microbial burden on common hospital surfaces through introduction of copper. *Journal of Clinical Microbiology*, 50 (7), 2217–2223.

Semeniuc, C.A., Pop, C.R., and Rotar, A.M., 2017. Antibacterial activity and interactions of plant essential oil combinations against Gram-positive and Gram-negative bacteria. *Journal of Food and Drug Analysis*, 25 (2), 403–408.

Senatore, F., 2002. Influence of harvesting time on yield and composition of the essential oil of a thyme (*Thymus pulegioides* L.) growing wild in Campania (Southern Italy). *Journal of Agricultural and Food Chemistry*, 44 (5), 1327–1332.

Seo, H.S., Beuchat, L.R., Kim, H., and Ryu, J.H., 2015. Development of an experimental apparatus and protocol for determining antimicrobial activities of gaseous plant essential oils. *International Journal of Food Microbiology*, 215, 95–100.

Shahina, Z., El-Ganiny, A.M., Minion, J., Whiteway, M., Sultana, T., and Dahms, T.E.S., 2018. *Cinnamomum zeylanicum* bark essential oil induces cell wall remodelling and spindle defects in *Candida albicans*. *Fungal Biology and Biotechnology*, 5 (1), 3.

Shelobolina, E.S., Walker, D.K., Parker, A.E., Lust, D. V., Schultz, J.M., and Dickerman, G.E., 2018. Inactivation of *Pseudomonas aeruginosa* biofilms formed under high shear stress on various hydrophilic and hydrophobic surfaces by a continuous flow of ozonated water. *Biofouling*, 34 (7), 826–834.

Shrivastava, S., Bera, T., Roy, A., Singh, G., Ramachandrarao, P., and Dash, D., 2007. Characterization of enhanced antibacterial effects of novel silver nanoparticles. *Nanotechnology*, 18 (22).

Siebert, T.E., Solomon, M.R., Pollnitz, A.P., and Jeffery, D.W., 2010. Selective determination of volatile sulfur compounds in wine by gas chromatography with sulfur chemiluminescence detection. *Journal of Agricultural and Food Chemistry*, 58 (17), 9454–9462.

Simões, M., Pereira, M.O., Machado, I., Simões, L.C., and Vieira, M.J., 2006. Comparative antibacterial potential of selected aldehyde-based biocides and surfactants against planktonic *Pseudomonas fluorescens*. *Journal of Industrial Microbiology and Biotechnology*, 33 (9), 741–749.

Simpkin, V.L., Renwick, M.J., Kelly, R., and Mossialos, E., 2017. Incentivising innovation in antibiotic drug discovery and development: progress, challenges and next steps. *The Journal of Antibiotics*, 70, 1087–1096.

Singer, A.C., Shaw, H., Rhodes, V., and Hart, A., 2016. Review of antimicrobial resistance in the environment and its relevance to environmental regulators. *Frontiers in Microbiology*, 7.

Singh, H.B., Srivastava, M., Singh, A.B., and Srivastava, A.K., 1995. Cinnamon bark oil, a potent fungitoxicant against fungi causing respiratory tract mycoses. *Allergy*, 50 (12), 995–999.

Slade, E.A., Thorn, R.M.S., Lovering, A.M., Young, A., and Reynolds, D.M., 2017. *In vitro* discrimination of wound-associated bacteria by volatile compound profiling using selected ion flow tube-mass spectrometry. *Journal of Applied Microbiology*, 123 (1), 233–245.

Smieszek, T., Pouwels, K.B., Christiaan, F., Dolk, K., Smith, D.R.M., Hopkins, S., Sharland, M., Hay, A.D., Moore, M. V, and Robotham, J. V, 2018. Potential for reducing inappropriate antibiotic prescribing in English primary care. *Journal of Antimicrobial Chemotherapy*, 73 (S2), 36-43STO.

Smith, D. and Španěl, P., 2011. Ambient analysis of trace compounds in gaseous media by SIFT-MS. *Analyst*, 136 (10), 2009–2032.

Solano, C., Echeverz, M., and Lasa, I., 2014. Biofilm dispersion and quorum sensing. *Current Opinion in Microbiology*, 18 (1), 96–104.

Soni, R., Sharma, G., and Jasuja, N.D., 2016. Essential oil yield pattern and antibacterial and insecticidal activities of *Trachyspermum ammi* and *Myristica fragrans*. *Scientifica*, 2016, 1428194.

Španěl, P., Dryahina, K., and Smith, D., 2006. A general method for the calculation of absolute trace gas concentrations in air and breath from selected ion flow tube mass spectrometry data. *International Journal of Mass Spectrometry*, 249 (250), 230–239.

Speight, J.G., 2019. *Natural Gas - A Basic Handbook*. 2nd ed. Oxford, UK: Gulf Professional.

Sriramulu, D.D., Lünsdorf, H., Lam, J.S., and Römling, U., 2005. Microcolony formation: A novel biofilm model of *Pseudomonas aeruginosa* for the cystic fibrosis lung. *Journal of Medical Microbiology*, 54 (7), 667–676.

Stepanović, S., Vuković, D., Dakić, I., Savić, B., and Švabić-Vlahović, M., 2000. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *Journal of Microbiological Methods*, 40 (2), 175–179.

Stojanović, G., Jovanović, O., Petrović, G., Mitić, V., Jovanović, V.S., and Jovanović, S., 2019. Composition of headspace volatiles and essential oils of three Thymus species. *Natural Product Communications*, 9 (11).

Storer, M.K., Hibbard-Melles, K., Davis, B., and Scotter, J., 2011. Detection of volatile compounds produced by microbial growth in urine by selected ion flow tube mass spectrometry (SIFT-MS). *Journal of Microbiological Methods*, 87 (1), 111–113.

Subhadra, B., Hwan Oh, M., and Hee Choi, C., 2016. Quorum sensing in Acinetobacter: with special emphasis on antibiotic resistance, biofilm formation and quorum quenching. *AIMS Microbiology*, 2 (1), 27–41.

Swamy, M.K., Akhtar, M.S., and Sinniah, U.R., 2016. Antimicrobial properties of plant essential oils against human pathogens and their mode of action: An updated review. *Evidence-based Complementary and Alternative Medicine*, 2016, 1–58.

Tak, J.H. and Isman, M.B., 2017. Penetration-enhancement underlies synergy of plant essential oil terpenoids as insecticides in the cabbage looper, Trichoplusia ni. *Scientific Reports*, 7, 1–11.

Tangjitjaroenkun, J., Chavasiri, W., Thunyaharn, S., and Yompakdee, C., 2012. Bactericidal effects and time-kill studies of the essential oil from the fruits of *Zanthoxylum limonella* on multi-drug resistant bacteria. *Journal of Essential Oil Research*, 24 (4), 363–370.

Tanwar, J., Das, S., Fatima, Z., and Hameed, S., 2014. Multidrug resistance: An emerging crisis. *Interdisciplinary Perspectives on Infectious Diseases*, 2014, 1–7.

Tenover, F.C., 2006. Mechanisms of antimicrobial resistance in bacteria. *American Journal of Infection Control*, 34 (5 SUPPL.).

Todd, J.D., Curson, A.R.J., Nikolaidou-Katsaraidou, N., Brearley, C.A., Watmough, N.J., Chan, Y., Page, P.C.B., Sun, L., and Johnston, A.W.B., 2010. Molecular dissection of bacterial acrylate catabolism - unexpected links with dimethylsulfoniopropionate catabolism and dimethyl sulfide production. *Environmental Microbiology*, 12 (2), 327–343.

Tong, S., Pan, J., Lu, S., and Tang, J., 2018. Patient compliance with antimicrobial drugs: A Chinese survey. *American Journal of Infection Control*, 46 (4), e25–e29.

Tongnuanchan, P. and Benjakul, S., 2014. Essential oils: extraction, bioactivities, and their uses for food preservation. *Journal of Food Science*, 79 (7), 1231–1249.

Toyofuku, M., Inaba, T., Kiyokawa, T., Obana, N., Yawata, Y., and Nomura, N., 2016. Environmental factors that shape biofilm formation. *Bioscience, Biotechnology, and Biochemistry*, 80 (1), 7–12.

Tracanna, V., de Jong, A., Medema, M.H., and Kuipers, O.P., 2017. Mining prokaryotes for antimicrobial compounds: from diversity to function. *FEMS Microbiology Reviews*, 41 (3), 417–429.

Tyagi, A.K. and Malik, A., 2010a. Antimicrobial action of essential oil vapours and negative air ions against *Pseudomonas fluorescens*. *International Journal of Food Microbiology*, 143 (3), 205–210.

Tyagi, A.K. and Malik, A., 2010b. *In situ* SEM, TEM and AFM studies of the antimicrobial activity of lemon grass oil in liquid and vapour phase against *Candida albicans*. *Micron*, 41 (7), 797–805.

Tyagi, A.K. and Malik, A., 2011. Antimicrobial potential and chemical composition of Eucalyptus globulus oil in liquid and vapour phase against food spoilage microorganisms. *Food Chemistry*, 126 (1), 228–235.

Tzortzakis, N.G., 2008. Impact of cinnamon oil-enrichment on microbial spoilage of fresh produce.

Ugur, A., Dagi, H., Ozturk, B., Tekin, G., and Findik, D., 2016. Assessment of in vitro antibacterial activity and cytotoxicity effect of Nigella sativa oil. *Pharmacognosy Magazine*, 12 (47), 471.

Umber, B.J., Shin, H.-W., Meinardi, S., Leu, S.-Y., Zaldivar, F., Cooper, D.M., and Blake, D.R., 2013. Gas signatures from *Escherichia coli* and *Escherichia coli*-inoculated human whole blood. *Clinical and Translational Medicine*, 2 (1), 13.

Utchariyakiat, I., Surassmo, S., Jaturanpinyo, M., Khuntayaporn, P., and Chomnawang, M.T., 2016. Efficacy of cinnamon bark oil and cinnamaldehyde on anti-multidrug resistant *Pseudomonas aeruginosa* and the synergistic effects in combination with other antimicrobial agents. *BMC Complementary and Alternative Medicine*, 16 (1), 158.

Veal, L., 1996. The potential effectiveness of essential oils as a Pediculus humanus capitis. *Complementary Therapies in Nursing and Midwifery*, 2 (4).

Velázquez-Nuñez, M.J., Avila-Sosa, R., Palou, E., and López-Malo, A., 2013. Antifungal activity of orange (*Citrus sinensis* var. Valencia) peel essential oil applied by direct addition or vapor contact. *Food Control*, 31 (1), 1–4.

Ventola, C.L., 2015. The antibiotic resistance crisis: causes and threats. *P&T Journal*, 40 (4), 277–83.

Vigan, M., 2010. Essential oils: Renewal of interest and toxicity. *European Journal of Dermatology*, 20 (6), 685–692.

Vohra, P. and Poxton, I.R., 2011. Efficacy of decontaminants and disinfectants against *Clostridium difficile*. *Journal of Medical Microbiology*, 60 (8), 1218–1224.

van Vuuren, S.F. and Viljoen, A.M., 2007. Antimicrobial activity of limonene enantiomers and 1,8-cineole alone and in combination. *Flavour and Fragrance Journal*, 22 (6), 540–544.

Wadhwani, T., Desai, K., Patel, D., Lawani, D., Bahaley, P., Joshi, P., Kothari Citation Wadhwani, V.T., and Kothari, V., 2012. Effect of various solvents on bacterial growth in context of determining MIC of various antimicrobials. *The Internet Journal of Microbiology*, 7 (1).

Walsh, S.E., Maillard, J.Y., Simons, C., and Russell, A.D., 1999. Studies on the mechanisms of the antibacterial action of ortho-phthalaldehyde. *Journal of Applied Microbiology*, 87 (5), 702–710.

Wang, R., Wang, R., and Yang, B., 2009. Extraction of essential oils from five cinnamon leaves and identification of their volatile compound compositions. *Innovative Food Science and Emerging Technologies*, 10 (2), 289–292.

Wang, T., Smith, D., and Španěl, P., 2002. Selected ion flow tube studies of the reactions of H3O+, NO+ and O2+ with the anaesthetic gases halothane, isoflurane and sevoflurane. *Rapid Communications in Mass Spectrometry*, 16 (19), 1860–1870.

Warnke, P.H., Becker, S.T., Podschun, R., Sivananthan, S., Springer, I.N., Russo, P.A.J., Wiltfang, J., Fickenscher, H., and Sherry, E., 2009. The battle against multi-resistant strains: Renaissance of antimicrobial essential oils as a promising force to fight hospital-acquired infections.

Journal of Cranio-Maxillofacial Surgery, 37 (7), 392–397.

Watson, F., Keevil, C.W., Wilks, S.A., and Chewins, J., 2018. Modelling vaporised hydrogen peroxide efficacy against mono-species biofilms. *Scientific Reports*, 8 (1).

Wen, P., Zhu, D.H., Wu, H., Zong, M.H., Jing, Y.R., and Han, S.Y., 2016. Encapsulation of cinnamon essential oil in electrospun nanofibrous film for active food packaging. *Food Control*, 59, 366–376.

Wentland, E.J., Stewart, P.S., Huang, C.T., and McFeters, G.A., 1996. Spatial variations in growth rate within *Klebsiella pneumoniae* colonies and biofilm. *Biotechnology Progress*, 12 (3), 316–321.

Whiley, H., Gaskin, S., Schroder, T., and Ross, K., 2018. Antifungal properties of essential oils for improvement of indoor air quality: A review. *Reviews on Environmental Health*, 33 (1), 63–76.

Williamson, K.S., Richards, L.A., Perez-Osorio, A.C., Pitts, B., McInnerney, K., Stewart, P.S., and Franklin, M.J., 2012. Heterogeneity in *Pseudomonas aeruginosa* biofilms includes expression of ribosome hibernation factors in the antibiotic-tolerant subpopulation and hypoxia-induced stress response in the metabolically active population. *Journal of Bacteriology*, 194 (8), 2062–2073.

Wilson, C., Lukowicz, R., Merchant, S., Valquier-Flynn, H., Caballero, J., Sandoval, J., Okuom, M., Huber, C., Brooks, T.D., Wilson, E., Clement, B., Wentworth, C.D., and Holmes, A.E., 2017. Quantitative and qualitative assessment methods for biofilm growth: a mini-review. *Journal of Engineering and Technology*, 6 (4).

Wolska, K.I., Grudniak, A.M., Rudnicka, Z., and Markowska, K., 2016. Genetic control of bacterial biofilms. *Journal of Applied Genetics*, 57 (2), 225–238.

Wu, J., Liu, H., Ge, S., Wang, S., Qin, Z., Chen, L., Zheng, Q., Liu, Q., and Zhang, Q., 2015. The preparation, characterization, antimicrobial stability and *in vitro* release evaluation of fish gelatin films incorporated with

cinnamon essential oil nanoliposomes. *Food Hydrocolloids*, 43, 427–435.

Xing, Y., Li, X., Xu, Q., Yun, J., Lu, Y., and Tang, Y., 2011. Effects of chitosan coating enriched with cinnamon oil on qualitative properties of sweet pepper (*Capsicum annuum* L.). *Food Chemistry*, 124, 1443–1450.

Yang, S.K., Yusoff, K., Mai, C.W., Lim, W.M., Yap, W.S., Lim, S.H.E., and Lai, K.S., 2017. Additivity vs. synergism: Investigation of the additive interaction of cinnamon bark oil and meropenem in combinatory therapy. *Molecules*, 22 (11).

Yangui, T., Bouaziz, M., Dhouib, A., and Sayadi, S., 2009. Potential use of Tunisian *Pituranthos chloranthus* essential oils as a natural disinfectant. *Letters in Applied Microbiology*, 48 (1), 112–117.

Yap, P.S.X., Yiap, B.C., Ping, H.C., and Lim, S.H.E., 2014. Essential Oils, A New Horizon in Combating Bacterial Antibiotic Resistance. *The Open Microbiology Journal*, 8 (1), 6–14.

Zabka, M., Pavela, R., and Prokinova, E., 2014. Antifungal activity and chemical composition of twenty essential oils against significant indoor and outdoor toxigenic and aeroallergenic fungi. *Chemosphere*, 112, 443–448.

Zaika, L.L., 1988. Spices and herbs: Their antimicrobial activity and its determination. *Journal of Food Safety*, 9 (2), 97–118.

Zaman, S. Bin, Hussain, A., Nye, R., Mehta, V., Taib Mamun, K., and Hossain, N., 2017. A Review on antibiotic resistance: Alarm bells are ringing. *Cureus*, 9 (6), 1–9.

Zhang, Y., Liu, X., Wang, Y., Jiang, P., and Quek, S., 2016. Antibacterial activity and mechanism of cinnamon essential oil against *Escherichia coli* and *Staphylococcus aureus*. *Food Control*, 59, 282–289.

Zheljazkov, V.D., Cantrell, C.L., Astatkie, T., and Jeliazkova, E., 2013. Distillation time effect on lavender essential oil yield and composition.

Journal of Oleo Science, 62 (4), 195–9.

Ziani, K., Chang, Y., McLandsborough, L., and McClements, D.J., 2011. Influence of surfactant charge on antimicrobial efficacy of surfactant-stabilized thyme oil nanoemulsions. *Journal of Agricultural and Food Chemistry*, 59 (11), 6247–6255.

Zouari-Bouassida, K., Trigui, M., Makni, S., Jlaiel, L., and Tounsi, S., 2018. Seasonal variation in essential oils composition and the biological and pharmaceutical protective effects of *Mentha longifolia* leaves grown in Tunisia. *BioMed Research International*, 2018, 1–12.

APPENDIX 1 – PUBLICATIONS

Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Rapid bactericidal effect of cinnamon bark essential oil against *Pseudomonas aeruginosa*

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Keywords
antimicrobial resistance, cinnamon, essential oils, GC-MS, multidrug resistance, plant extract, *Pseudomonas aeruginosa*, time-kill.

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2019/0562: received 31 March 2019, revised 27 November 2019 and accepted 28 November 2019

doi:10.1111/jam.14538

Abstract

Aims: This study aimed to identify the most effective antimicrobial agent from a selection of essential oils (EO) and investigate its bactericidal properties against *Pseudomonas aeruginosa*.

Methods and Results: The disc diffusion assay and minimal inhibitory/bactericidal concentration tests were used to identify antimicrobial potential. Several oils exhibited antimicrobial effects at concentrations as low as 0·03% (v/v). Significantly, cinnamon (*Cinnamomum zeylanicum*) bark EO exhibited a broad-spectrum activity against Gram-negative and Gram-positive bacteria and showed bacteriostatic and bactericidal effects against *P. aeruginosa* PAO1 at 0·125% (v/v) and all other tested organisms, including known multidrug resistant species. Time-kill assays and metabolic activity tests showed cinnamon oil to exhibit rapid killing, with bactericidal activity observed in ≤6 min at ≥0·5% (v/v). Furthermore, scanning electron microscopy and a membrane permeability assay indicated damage to membrane integrity, loss of turgor and cell collapse.

Conclusion: Cinnamon bark EO is a broad-spectrum antimicrobial agent capable of rapid killing at low concentrations.

Significance and Impact of the Study: This study provides a sound basis for further investigation of the potential of cinnamon bark EO as an alternative to conventional antimicrobial products due to its fast-acting bactericidal properties at low concentrations.

Introduction

Approximately 100 000 tonnes of antibiotics are manufactured globally per year (Nikaido 2009). Not only have bacteria developed an antimicrobial resistance (AMR), but also many strains have become resistant to multiple antibiotics and chemotherapeutic agents, termed multidrug resistance (MDR) (Nikaido 2009). Antimicrobial resistance contributes to unsuccessful management of bacterial pathogens, higher infection spread and perseverance (Tanwar *et al.* 2014). In the European Union, MDR infections are responsible for approx. 25 000 patient deaths per year, and result in extra health care costs and productivity losses in the EU reported to cost at least 1·5 billion euros each year (Department of Health 2016). With the rising global threat of MDR, developing new therapeutic agents, improving the existing infection control tools and antimicrobial stewardship programs are of huge importance.

Potential options for novel antimicrobials include the use of natural compounds such as those derived from plants (essential oils (EOs), phenolics, lectins and polyacetylenes), animals (lactoferrin, chitosan and lysozymes), bacteria (bacteriocin, reuterin), algae and fungi (Cowan 1999; Gyawali and Ibrahim 2014). Recent approaches include exploring the peptide-based antibiotics (Roshan *et al.* 2018) and sequencing the prokaryote genomes to discover novel antimicrobial molecules (Tracanna *et al.* 2017).

Essential oils are compounds produced by plants, and are known to have activity against both Gram-negative and Gram-positive bacteria in both motile and sessile states (Millezi *et al.* 2016). EOs have been used in medicine for many years and are widely reported as possessing

antibacterial, antifungal and anti-plasmodial properties (Utchariyakiat *et al.* 2016). However, of the approx. 3000 EOs known, only approx. 300 are used commercially (Ghabraie *et al.* 2016). While it is thought that the majority of EOs act on the cell wall and membrane of bacteria (Faleiro 2011), studies assessing the mode of action of individual oils are required. Furthermore, very few studies exploring the antimicrobial activities of EOs investigate their potential for rapid killing, that is, under 10 min contact time. Friedman *et al.* (2004) investigated the activities of EOs and their components against *Escherichia coli* O157:H7 with incubation time ≥5 min, and Tangjitjaroenkun *et al.* (2012) studied the antimicrobial effects of EO from *Zanthoxylum limonella* with incubation time ≥3 min. However, no studies to date have reported rapid killing against *Pseudomonas aeruginosa* using EOs.

Therefore, the purpose of this study was to assess the effects of a selection of EOs against a panel of known pathogenic bacteria. In addition, EOs exhibiting the greatest antimicrobial effects were investigated for its rapid bactericidal potential and mode of action against *P. aeruginosa*.

Materials and methods

Essential oils

Fifteen EOs were used, and included EOs from: bergamot (*Citrus bergamia*), cinnamon (*Cinnamomum zeylanicum*) bark and leaf, clove (*Eugenia caryophyllus*), grapefruit (*Citrus paradisi*), lavender (*Lavandula angustifolia*), lemon (*Citrus limonum*), lemongrass (*Cymbopogon citratus*), lime (*Citrus aurantifolia*), manuka (*Leptospermum scoparium*), sweet orange (*Citrus sinensis*), rose geranium (*Pelargonium graveolens*), rosemary (*Rosmarinus officinalis*), rosewood (*Dalbergia sissoo*), tea tree (*Melaleuca alternifolia*) and thyme (*Thymus vulgaris*). All of these oils were donated by Amphora Aromatics Ltd, Bristol, UK for research purposes, with the exception of cinnamon leaf oil, which was sourced from Natural by Nature Ltd (Bedfordshire, UK). All the oils used in this study were extracted by steam distillation as indicated by the suppliers.

Test organisms and standardisation of overnight cultures

The bacteria tested were obtained from the microbiology culture collection at the University of the West of England, Bristol, UK. The strains used were: *E. coli* (ATCC 23848), *E. coli* (NCTC 9001), *Staphylococcus aureus* (ATCC 6358), *S. aureus* (NCTC 12981), *Pseudomonas aeruginosa* (PAO1), *P. aeruginosa* (NCTC 8505), hospital acquired methicillin-resistant *S. aureus* (MRSA) isolate, methicillin-susceptible *Staphylococcus aureus* (NCTC 13297), *Acinetobacter baumannii* (NCTC 12156) and *A. baumannii* (ATCC 17978). The bacteria were stored on beads (Microbank; Pro Lab Diagnostics, Canada) at −80°C and revived on nutrient agar (Oxoid, Hampshire, UK) slopes at 37°C for 24 h. Overnight broth cultures were prepared using one to three colonies of bacteria added to 10 ml of tryptone soy broth (TSB; Oxoid) and incubated at 37°C for 24 h. Cultures were standardized by diluting with TSB and measuring OD at 600 nm to obtain a reading of 0·08–0·1 (McFarland 0·5), giving a standardized inoculum of approx. $1–2 \times 10^8$ CFU per ml.

Disc diffusion assay

Screening of EOs was performed using a paper disc diffusion approach adapted from the Clinical and Laboratory Standards Institute (CLSI) Antimicrobial Susceptibility Testing (AST) Standards (Clinical and Laboratory Standards Institute 2015). Mueller-Hinton agar (MHA) plates (Oxoid) were inoculated with 100 µl of standardized culture for each test bacterium, spread evenly over the entire surface of the agar using a sterile cotton swab by swabbing in three directions (Andrews 2007). The inoculum applied to each plate was approx. $1–2 \times 10^7$ CFU per ml. A volume of 10 µl of EO was used to saturate a set of 6-mm diameter filter paper discs (Whatman; Sigma Alrdich, Dorset, UK), one of which was then placed onto the centre of each inoculated plate. Blank discs were used as a negative control and discs containing 30 µg gentamicin was used as a positive reference. Plates were incubated at 37°C for 24 h and antibacterial activity was assessed by measuring the diameter of the zones of inhibition in mm using Vernier callipers. Each experiment was repeated on three separate occasions, with a minimum of three replicates in each repeat experiment.

Determination of minimal inhibitory concentration and minimum bactericidal concentration

The minimal inhibitory concentration (MIC) was determined by the broth microdilution method as outlined by the CLSI (2012) and adapted from previously described methods (Cao *et al.* 2009). A 16% (v/v) stock solution of EO was prepared in TSB containing 10% (v/v) dimethylsulphoxide (DMSO; Sigma Aldrich) which is a solvent serving a dispersal agent (Kačániová *et al.* 2017). Twofold dilutions of this stock solution were made in TSB to produce a range of EO concentrations from 0·015 to 8% (v/v), with DMSO concentrations of 0·0098–5% (v/v) respectively. One hundred microliters of each EO

concentration was added to wells of a 96-well microtitre plate. A standardized overnight culture was diluted 1/150 with TSB, and 100 µl of this was added to each well of the microtitre plate. Final concentrations of EO ranged from 0·007 to 4% (v/v) with DMSO concentrations of 0·0049 to 2·5% (v/v) respectively. Final cell density of the inoculum was approx. 5×10^5 CFU per ml. Microplate wells of EO dilutions without bacteria, and TSB and DMSO alone, were used as negative controls, and plates were incubated at 37°C for 24 h. MIC values were determined at the well with the lowest concentration of EO where no visible microbial growth was observed. Each concentration of EO had three replicates and was repeated four times.

Minimum bactericidal concentration (MBC) was determined by spot inoculation similar to the methods outlined by Adukwu *et al.* (2012). Briefly, 10 µl was pipetted from the microtitre wells showing no growth onto MHA, and incubated for 24 h at 37°C. Each concentration of EO had three replicates and was repeated four times. MBC values were determined at the lowest concentration of EO where no growth was observed.

Gas chromatography–mass spectrometry analysis

Following the determination of MICs and MBCs, cinnamon bark EO demonstrated broad-spectrum activity against all tested organisms, including *P. aeruginosa* at <4% (v/v). Thus, this oil was selected for testing rapid bactericidal effects against *P. aeruginosa*.

Gas chromatography–mass spectrometry analysis (GC-MS) of cinnamon bark EO was performed using the methods adapted from Adukwu *et al.* (2012). Cinnamon leaf EO was also analysed as a comparator to the bark EO to identify the differences in the components as these EOs are obtained from different parts of the same plant. GC-MS analysis used an Agilent 6890N Network Gas Chromatograph system and 5973 Network Mass Selective Detector (Agilent Technologies, Santa Clara, CA). Samples were dissolved in acetone with a 1 µl injection volume. Inlet temperature was 300°C with a split ratio of 10 : 1. Temperature of transfer line was 300°C and solvent delay was 3 min. The carrier gas was helium with a column flow rate of 1 ml min^{-1} and operating at constant flow. The oven temperature started at 50°C and was held for 2 min, then increased until 280°C was reached at a rate of 10°C per min, with a total run time of 25 min. Compound separation was achieved with a HP-5MS column (30 m × 0·25 mm × 0·25 µm). An electron impact ion source at 230°C and a quadrupole mass analyser at 150°C were used. Electron ionization mass spectrometric data were collected between 30 and 550 m/z. Oil components were identified by comparing the mass spectral patterns with those from the spectra from the National Institute of Standards and Technology (NIST) library (MS Search 2.0) provided by the software of the GC-MS system.

Time-kill assay

Time-kill kinetics were determined for cinnamon bark EO against *P. aeruginosa* PAO1 using the methods adapted from Carson *et al.* (2002). Six 50 ml conical flasks containing 15 ml of TSB were inoculated with 50 µl of standardized *P. aeruginosa* PAO1 overnight culture to achieve approx. 5×10^5 CFU per ml. A 100 µl aliquot was removed from each flask and added to 9·9 ml of TSB, diluted serially and plated onto MHA to confirm starting inoculum density, representing the zero min time point (T0). Table 1 shows the range of volumes of EO and DMSO added to the flasks to achieve a series of concentrations for the time-kill assay. The concentration of cinnamon bark EO ranged from 0·125% (v/v; one × MIC) to 2% (v/v; 16 × MIC), with a negative control flask containing DMSO alone. After the addition of EO/DMSO, the flasks were vortexed and incubated stationary at room temperature. At each time point, the flasks were vortexed again and aliquots were removed at 2, 4, 6, 10 and 30 min and at 1, 2, 4, 6 and 24 h, diluted serially and plated onto MHA. All plates were incubated at 37°C for 24 h before enumeration of the colonies. An antimicrobial compound is considered bactericidal if a ≥99·9% decrease in the initial inoculum (i.e. a 3-log reduction) is observed, as described by the CLSI (1999).

Metabolic activity assay

The metabolic activity assay of triphenyltetrazolium chloride (TTC) was performed to investigate the effect of EO on metabolic activity, as described by Ahmed (2013), during the time-kill assay. One hundred microlitre aliquots of the treated organisms from each time point was transferred to wells of a 96-well plate. A 5 µl volume of sterile 0·035 mol l^{-1} aqueous solution of TTC (Sigma Alrdich) was added to each well and plates were wrapped in aluminium foil and incubated at 37°C with orbital shaking at 120 rev min^{-1} for 24 h. TSB containing no bacteria was used as a negative control. The presence of viable bacterial cells was indicated by the reduction of the yellow TTC to a red colour. OD of the wells was measured using a TECAN Infinite® 200 PRO plate reader at 595 nm. Data were normalized to T0 and expressed as a percentage relevant to this time point. All assays were performed in triplicate on three separate occasions.

Table 1 Antimicrobial activity of 16 essential oils using disc diffusion assay. Values are mean inhibition zone (mm) ±SE of three independent experients and nine replicates ($n = 3$)

Essential oils	*Escherichia coli* B ATCC 23848	*E. coli* NCTC 9001	*Staphylococcus aureus* ATCC 6358	*S. aureus* NCTC 12981	Hospital acquired MRSA isolate	MSSA NCTC 13297	*Pseudomonas aeruginosa* PAO1	*P. aeruginosa* NCTC 8505	*Acinetobacter baumannii* NCTC 12156	*A. baumannii* ATCC 17978
Bergamot	11·0 ± 0·4†	–	7·0 ± 0·0	10·7 ± 0·4	6·4 ± 1·6	5·0 ± 1·3	–	–	5·1 ± 1·3	4·7 ± 1·2
Cinnamon bark	36·2 ± 1·2†	26·6 ± 0·7†	30·8 ± 0·5†	28·7 ± 0·5	35·4 ± 1·8†	27·7 ± 1·1	17·3 ± 0·7	16·7 ± 1·4	28·6 ± 0·7†	24·7 ± 0·8†
Cinnamon leaf	18·2 ± 0·4	13·2 ± 0·2	15·4 ± 0·2	17·9 ± 0·7	14·8 ± 0·2	12·9 ± 0·3	–	8·7 ± 0·3	17·9 ± 0·2	16·0 ± 0·0
Clove	20·4 ± 0·6	13·2 ± 0·4	15·0 ± 0·7	20·1 ± 0·8	15·3 ± 1·1	13·3 ± 0·1	5·2 ± 1·3	5·2 ± 1·3	18·1 ± 0·4†	15·8 ± 0·5
Grapefruit	13·6 ± 0·5	8·5 ± 1·4	9·8 ± 0·3	11·2 ± 0·7	11·0 ± 0·4	10·3 ± 0·7	–	–	2·3 ± 1·2	–
Lavender	12·3 ± 0·7	7·7 ± 0·3	14·7 ± 1·7	12·1 ± 0·4	11·6 ± 1·0	9·8 ± 0·2	–	–	8·9 ± 0·5	9·1 ± 0·3
Lemon	2·9 ± 1·0	–	9·0 ± 0·9	6·4 ± 0·7	6·2 ± 0·8	4·7 ± 2·1	–	–	–	–
Lemongrass	32·6 ± 1·0†	10·1 ± 0·2	29·2 ± 0·9	27·6 ± 0·7	36·9 ± 0·8†	26·8 ± 2·1	–	–	14·2 ± 1·5	24·6 ± 2·6
Lime	14·7 ± 0·8	10·6 ± 1·1	11·1 ± 0·3	12·8 ± 0·6	10·1 ± 0·8	10·1 ± 0·7	–	–	12·0 ± 0·6	11·6 ± 0·2
Manuka	7·3 ± 0·3	5·3 ± 1·5	19·7 ± 4·0	20·9 ± 3·9	21·6 ± 4·7	17·3 ± 3·7	–	–	5·0 ± 1·2	–
Sweet orange	9·1 ± 1·9	3·0 ± 1·3	11·3 ± 3·2	7·9 ± 3·5	9·3 ± 5·4	7·4 ± 4·3	–	–	2·3 ± 1·3	–
Rose geranium	18·3 ± 0·3	11·3 ± 0·3	17·7 ± 0·6	15·5 ± 0·8	12·3 ± 0·4	13·4 ± 0·3	5·3 ± 1·0	–	8·6 ± 0·2	11·4 ± 0·6
Rosemary	15·6 ± 1·2	16·1 ± 0·3	16·8 ± 0·7	19·5 ± 2·1	19·2 ± 1·4†	17·4 ± 1·1	–	–	16·5 ± 1·3	14·7 ± 1·4
Rosewood	21·7 ± 2·0	14·7 ± 0·7	16·4 ± 2·1	20·3 ± 1·0	16·2 ± 0·4	14·0 ± 0·7	–	–	20·04 ± 1·1†	16·6 ± 0·5
Tea tree	16·7 ± 0·6	16·4 ± 0·6	23·4 ± 1·7	22·4 ± 2·1	17·2 ± 0·5	13·5 ± 1·0	–	–	18·9 ± 1·2†	16·6 ± 0·7
Thyme	46·2 ± 4·8†	37·2 ± 1·1†	48·2 ± 2·1†	36·4 ± 2·2	35·4 ± 3·0†	35·8 ± 2·8†	21·2 ± 2·0	17·1 ± 4·5	41·6 ± 2·1†	36·4 ± 5·3†
Gentamicin*	24·4 ± 0·2	22·7 ± 0·2	27·7 ± 0·2	35·3 ± 0·2	12·4 ± 0·2	26·4 ± 0·2	20·3 ± 0·2	26·0 ± 0·0	12·1 ± 0·1	19·6 ± 0·2

–, no activity.
*Gentamicin disc (30 μg).
†Value is significantly greater than gentamicin positive reference ($P < 0.05$).

Scanning electron microscopy

Scanning electron microscopy (SEM) was used to assess the morphological effects on *P. aeruginosa* PAO1 after treatment with cinnamon bark EO at 2% (v/v) for 10 min. Both untreated and treated samples were prepared for SEM using the method reported by Murtey and Ramasamy (2016) and adjusted as indicated below. Briefly, samples were allowed to settle on squares of poly-L-lysine coated microscope slides (Sigma Alrdich) in Petri dishes lined with damp filter paper for 1 h. The attached cells were fixed by immersion in 0·424 mol l^{-1} glutaraldehyde (Sigma Alrdich) in 0·1 mol l^{-1} phosphate buffer for 30 min, washed with the same buffer three times and then dehydrated through a series of increasing ethanol concentrations (35, 50, 75, 95, 95, 100, 100% v/v) for 10 min each, followed by hexamethyldisilizane (Sigma Alrdich) for 10 min two times. The squares were mounted on aluminium stubs and coated with gold in a sputter coater and viewed using a scanning electron microscope (FEI Quanta 650 FEG, Sigma Aldrich).

Release of nucleic acids and proteins

The release of 260 nm absorbing nucleic acids and 280 nm absorbing proteins from *Ps. aeruginosa* PAO1 after treatment with cinnamon bark EO was measured according to methods described by Miksusanti *et al.* (2008), with some adaptations. A 50 ml conical flask containing 15 ml of cinnamon bark EO at either 2% (v/v) or 1·25% (v/v) in TSB with 1·25% (v/v) or 0·08% (v/v) of DMSO, respectively, was inoculated with 50 µL of standardized overnight *P. aeruginosa* PAO1 culture. The flask was vortexed for 30 s, incubated at room temperature for 2 h and then a 1 ml aliquot was removed and filtered with 0·45 µm filter. The absorbance of this filtrate was measured at 260 and 280 nm using a Jenway 6305 UV-Vis spectrophotometer (Cole-Parmer, Saint Neots, UK). Negative controls were treated with DMSO (1·25% v/v) alone. The absorbance of filtrate from controls without adding the culture was deducted from the absorbance of the respective samples with EO added. Experiments were performed in triplicate on three separate occasions and the results are expressed as mean OD of nucleic acids (260 nm) and protein (280 nm).

Data analysis

All data were analysed using Microsoft Excel 2016 (Microsoft Corporation, Redmond, CA) and GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla CA). An unpaired *t*-test was applied when the results of treated and untreated samples were assessed following the disc diffusion assay while the one-way ANOVA and Dunnett's multiple comparison were used to assess any differences between treatments and control following the membrane permeability assay.

Results

Disc diffusion

The results from the antimicrobial susceptibility screening assay demonstrated that all the EOs used in this study were antimicrobial against most, if not all, bacteria tested (Table 1). Three of the EOs, cinnamon bark, clove and thyme, produced zones of inhibition against all strains tested, including *P. aeruginosa* PAO1, demonstrating a broad-spectrum activity. Inhibition zones shown by thyme and cinnamon bark EO were significantly larger ($P < 0·05$) than that of the gentamicin antibiotic reference in 70 and 60% of the strains used respectively. Thyme produced zones of inhibition ranging from 17·1 to 48·2 mm, while cinnamon bark produced zones ranging from 16·7 to 36·2 mm. Rose geranium EO showed activity against all strains excluding *P. aeruginosa* NCTC 8505, producing zones ranging from 5·3 to 18·3 mm. Cinnamon leaf EO showed activity against all strains excluding *P. aeruginosa* PAO1, producing zones ranging from 8·7 to 18·2 mm. Lavender, lemongrass, lime, rosemary, rosewood and tea tree EOs showed effects against both Gram-positive and Gram-negative bacteria, excluding the *Pseudomonas* species. Of these oils, lemongrass produced the largest zones of inhibition, ranging from 10·1 to 36·9 mm. The smallest zones of inhibition were produced by bergamot, grapefruit, lemon, manuka and sweet orange EOs. Blank disc negative controls did not affect the growth of any of the strains. In accordance with the breakpoint tables for inhibition zone interpretation published by the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2015), all bacteria in this study were sensitive to gentamicin with the exception of MRSA and *A. baumannii* NCTC 12156.

Determination of MIC and MBC

Nine EOs were selected, based on their antimicrobial activity shown in the disc diffusion assay, to determine their MIC and MBC (Table 2). The data show that rose geranium, rosewood and tea tree produced MICs between 0·5 and 4% or greater (v/v) against most strains and that MBC values for these are either equal or double the MIC value. Although manuka EO demonstrated a MIC as low as 0·06% (v/v), the inhibitory effects were only against Gram-positive *S. aureus* strains. EO of manuka did not demonstrate any bactericidal action at <4% (v/v). EOs of

Table 2 The antimicrobial activity of nine essential oils, determined by the broth microdilution method to find the minimal inhibitory concentration and minimum bactericidal concentration. The results shown are in (% v/v) and from four independent experiments and a total of 12 replicates ($n = 4$)

	Escherichia coli B ATCC 23848		*E. coli* NCTC 9001		*Staphylococcus aureus* ATCC 6358		*S. aureus* NCTC 12981		Hospital acquired MRSA isolate		MSSA NCTC 13297		*Pseudomonas aeruginosa* PAO1		*P. aeruginosa* NCTC 8505		*Acinetobacter baumannii* NCTC 12156		*A. baumannii* ATCC 17978	
Essential oils	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Cinnamon bark	0·03	0·125	0·06	0·125	0·06	0·125	0·03	0·06	0·015	0·015	0·03	0·125	0·125	0·125	0·25	0·25	0·03	0·125	0·06	0·125
Cinnamon leaf	0·125	0·125	0·125	0·125	0·125	0·125	0·125	0·125	0·125	0·25	0·125	0·25	>4·0	>4·0	>4·0	>4·0	0·125	0·125	0·125	0·125
Clove	0·125	0·125	0·125	0·125	0·125	0·25	0·125	0·25	0·25	0·25	0·25	0·25	>4·0	>4·0	>4·0	>4·0	0·125	0·125	0·125	0·125
Lemongrass	0·25	0·25	1·0	1·0	0·25	0·25	0·25	0·25	0·12	0·25	0·25	0·25	>4·0	>4·0	>4·0	>4·0	1·0	1·0	0·5	1·0
Manuka	>4·0	>4·0	>4·0	>4·0	0·06	>4·0	0·06	>4·0	0·06	>4·0	0·06	>4·0	>4·0	>4·0	>4·0	>4·0	>4·0	>4·0	>4·0	>4·0
Rose geranium	1·0	2·0	>4·0	>4·0	0·5	1·0	1·0	2·0	1·0	2·0	1·0	2·0	>4·0	>4·0	>4·0	>4·0	2·0	4·0	>4·0	>4·0
Rosewood	0·5	1·0	1·0	2·0	1·0	2·0	1·0	2·0	1·0	2·0	1·0	2·0	>4·0	>4·0	>4·0	>4·0	0·5	1·0	1·0	1·0
Tea tree	1·0	1·0	1·0	1·0	2·0	>4·0	>4·0	>4·0	>4·0	>4·0	>4·0	>4·0	>4·0	>4·0	>4·0	>4·0	4·0	4·0	2·0	2·0
Thyme	0·125	0·25	0·25	0·25	0·125	0·50	0·25	0·25	0·25	0·50	0·25	0·50	>4·0	>4·0	>4·0	>4·0	0·125	0·125	0·25	0·25

thyme, lemongrass, cinnamon leaf and clove presented MICs and MBCs of ≤1·0% (v/v) for all strains of bacteria excluding the *Ps. aeruginosa* strains. The only EO which demonstrated a broad-spectrum antimicrobial activity in this investigation was cinnamon bark EO. All the bacteria tested including the *Pseudomonas* sp. were inhibited by cinnamon bark EO at ≤0·25% (v/v), which also demonstrated bactericidal activity at the same concentration.

Gas chromatography–mass spectrometry analysis

Components of cinnamon bark and cinnamon leaf EO and their percentage composition are reported in Table 3. There were noticeably more components from the cinnamon leaf EO than from cinnamon bark EO with 20 components identified from the leaf EO in comparison to seven components identified from the bark EO. The major components from either EO were different and represented approx. 85% of the whole oil with the rest of the components representing the remaining 15% of the EO. For the cinnamon bark EO, the main component was Σ-cinnamaldehyde representing approx. 85·312% of the EO whereas from the cinnamon leaf EO, eugenol was identified as the major component representing 84·481% of the whole EO.

Table 3 Percentage (%) composition of components within cinnamon bark and cinnamon leaf essential oils after gas chromatography–mass spectrometry (GCMS) analysis

	Percentage (%) of total composition of oil	
Component	Cinnamon bark	Cinnamon leaf
Σ-Cinnamaldehyde	85·312	0·185
D-limonene	4·665	–
Eucalyptol	3·823	0·160
Eugenol	3·366	84·481
Benzyl benzoate	1·935	2·752
β-Linalool	0·897	1·627
Caryophyllene	–	3·224
Acetyleugenol	–	2·191
α-Pinene	–	0·982
Camphene	–	0·282
β-Terpinene	–	0·242
α-Phellandrene	–	0·957
3-Carene	–	0·04
Isoterpinolene	–	0·062
m-Cymene	–	0·638
2-Thujene	–	0·201
p-Menth-1-en-8-ol	–	0·122
Copanene	–	0·633
Cinnamylalcohol acetate	–	0·640
α-Caryophyllene	–	0·434
Carypyllene oxide	–	0·141
Total	99·998	99·994

Time-kill assay—cinnamon bark EO against *P. aeruginosa* PAO1

A time-kill assay was performed to evaluate the bactericidal potential of cinnamon bark EO against *P. aeruginosa* PAO1 (Fig. 1). At the MIC, 0·125% (v/v), cinnamon bark EO was bactericidal (3-log reduction) against *P. aeruginosa* PAO1 after 6 h. At concentrations twofold and fourfold of the MIC, 0·25% (v/v) and 0·5% (v/v), a 99·999% (5-log) kill, were achieved within 30 and 6 min respectively. At the higher EO concentrations of 1 and 2% (v/v), 5-log reduction was achieved within 2 min.

Metabolic activity assay

Reduction of TTC from a yellow to red colour was observed in microtitre wells containing viable *P. aeruginosa*, but no colour change was observed when cells were nonviable (Fig. 2). Negative control wells with TSB containing no bacteria remained clear with no colour change. All results obtained from the TTC assay reflected results from the time-kill assay are shown in Fig. 1. *Pseudomonas aeruginosa* continued to show metabolic activity after 10 min of contact time when cinnamon bark EO was at 0, 0·125 and 0·25% (v/v), with some activity after 4 min of contact time with EO at 0·5% (v/v), but no activity after 6 min. Metabolic activity had ceased after 2 min of contact time at concentrations equal or greater to 1% (v/v).

Scanning electron microscopy

Electron micrographs of both untreated and cinnamon bark EO-treated *P. aeruginosa* cells are presented in Figs 3 and 4. The untreated cells (Fig. 3a,b) show a turgid structure with a particulate surface, and are of expected lengths (1–5 μm). In contrast, cells treated with cinnamon bark EO showed an altered morphology (Fig. 4a,b), with cells appearing collapsed with loss of turgidity and

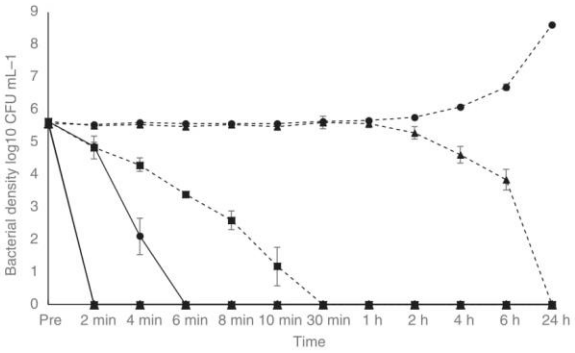

Figure 1 Time-kill of *Pseudomonas aeruginosa* PAO1 in the presence of cinnamon bark essential oil. Essential oil concentrations: (-●-) 0% (v/v); (-▲-) 0·125% (v/v); (-■-) 0·25% (v/v); (—●—) 0·5% (v/v); (—▲—) 1·0% (v/v); (—■—) 2·0% (v/v). Bars show SE. N = 11 for 0% EO control sample, N = 3 for remaining samples.

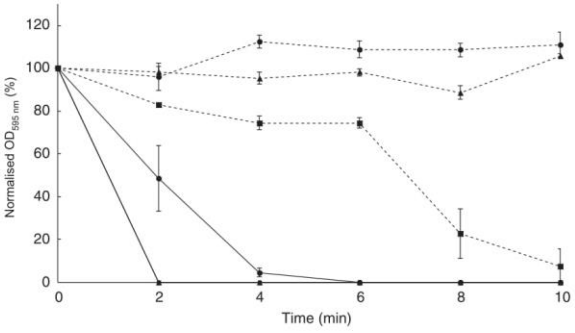

Figure 2 OD_{595nm} data for triphenyltetrazolium chloride assay after treating *Pseudomonas aeruginosa* PAO1 with cinnamon bark essential oil, normalized to T0 and expressed as a percentage. Essential oil concentrations: (-●-) 0% (v/v); (-▲-) 0·125% (v/v); (-■-) 0·25% (v/v); (—●—) 0·5% (v/v); (—▲—) 1·0% (v/v); (—■—) 2·0% (v/v). Bars show SE (n = 9).

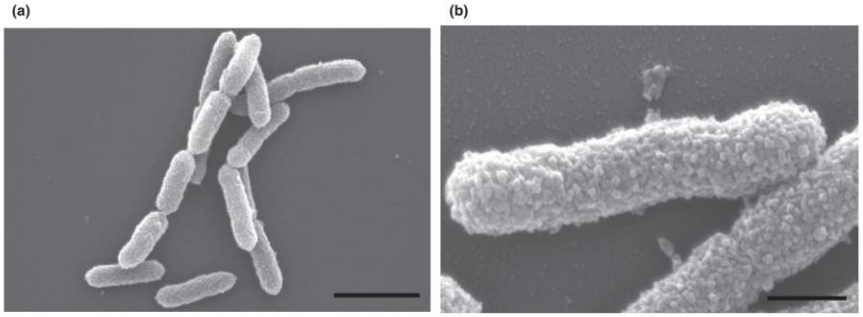

Figure 3 Scanning electron micrograph showing untreated *Pseudomonas aeruginosa* PAO1 cells. (a) Scale bar 2 μm. (b) Scale bar 2 μm.

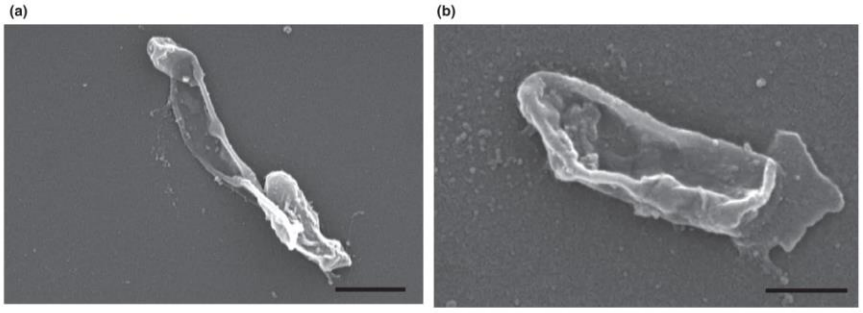

Figure 4 Scanning electron micrograph showing *Pseudomonas aeruginosa* PAO1 treated with 2% cinnamon bark essential oil for 10 min. (a) Scale bar 500 nm (b) Scale bar 1 μm.

few surface particles after 10 min contact time with 2% (v/v) cinnamon bark EO.

Release of nucleic acids and proteins

Relative OD of filtrate from *P. aeruginosa* untreated or treated with cinnamon bark EO measured at 260 and 280nm are displayed in Fig. 5a,b respectively. An increased OD_{260nm} reading signifies an increase in nucleic acids, while an increase in OD_{280nm} indicates an increase in proteins (Miksusanti *et al.* 2008) released from bacterial cells. An increase was seen in both OD_{260nm} and OD_{280nm} when bacteria were treated with cinnamon bark EO at either 0·125 or 2% (v/v) when compared to the untreated cultures. There was a significant difference ($P \leq 0{\cdot}01$) between readings for the untreated bacteria and bacteria treated with 2% (v/v) EO.

DISCUSSION

This study examined the effect of EOs against pathogenic bacteria, some of which exhibit AMR. Disc diffusion assays using EOs at 100% (v/v) concentration demonstrated antimicrobial activity against the selected panel of Gram-positive and Gram-negative bacteria. The use of an established antibiotic (gentamicin) provided a reference against which the sensitivity of the bacteria was assessed. Gentamicin sensitivity was observed in all tested bacteria with the exception of MRSA and *A. baumannii* NCTC 12156. Zones of inhibition breakpoints for MRSA and *A. baumannii* treated with gentamicin are 18 and 17 mm respectively.

The disc diffusion assay showed that the oils showing the greatest inhibitory effects were thyme, cinnamon bark and lemongrass. Of these, thyme EO was most effective,

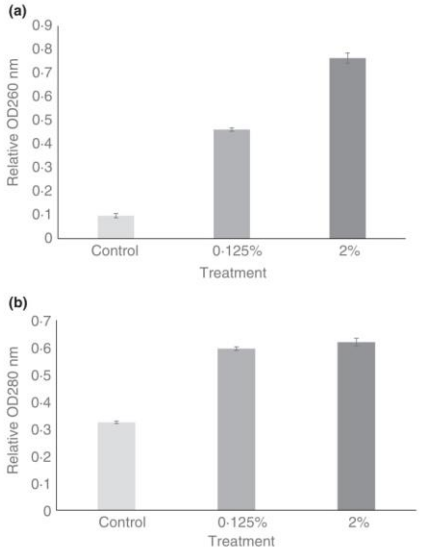

Figure 5 Assessment of nucleic acid and protein release from bacteria after treatment with cinnamon bark essential oil at 0·125 and 2% (v/v), and the untreated *Pseudomonas aeruginosa* control culture. (a) Relative OD_{260nm} demonstrating nucleic acid release and (b) relative OD_{280nm} of filtrate. This experiment was carried out on three separate occasions ($N = 3$) with the bars showing SE.

an observation reported previously by Semeniuc *et al.* (2017) where it was most effective when compared to three other oils. Although the MRSA strain used was resistant to gentamicin in this study, cinnamon bark, lemongrass, manuka, rosemary and thyme EOs all produced zones >18 mm. *Acinetobacter baumannii* NCTC 12156 also showed resistance against gentamicin, although tea tree, thyme, rosewood, clove, cinnamon bark and cinnamon leaf EOs all produced zones ≥17 mm. These data are in line with published reports (Doran *et al.* 2009; Adukwu *et al.* 2012; Priti and Shridhar 2012; Yap *et al.* 2014; Sakkas *et al.* 2016), which indicate that EOs are capable of working effectively against bacteria resistant to commercial antibiotics.

Essential oils of lemon, lemongrass, manuka, sweet orange and tea tree were more effective against the Gram-positive bacteria in comparison to the Gram-negative bacteria. Lemongrass and manuka in particular were shown to have lower MICs/MBCs for *S. aureus* when compared to other bacteria tested. These findings are in agreement with the studies that found manuka EO to be more effective against *S. aureus* compared to *E. coli* and *P. aeruginosa* (Porter and Wilkins 1998), and studies where lemongrass EO also showed good effect against Gram-positive bacteria (Hammer *et al.* 1999). In the present study, cinnamon bark at very low concentrations (i.e. 0·015% v/v) was bactericidal against MRSA, a finding which is supported by Cui *et al.* (2016), where cinnamon oil was found to be bactericidal against MRSA at 0·2 μg ml^{-1}.

Cinnamon leaf, clove, lemongrass, rosewood and thyme oils at <4% (v/v) were bactericidal against all the bacteria, excluding *Pseudomonas* species. A study by Kavanaugh and Ribbeck (2012) also reported a lack of bactericidal activity from EOs of clove, thyme and tea tree against *P. aeruginosa* sp. at concentrations ≤4% (v/v). Different studies have shown that EOs are more effective against Gram-positive bacteria (Lodhia *et al.* 2009), which is supported by some of the results presented here. However, equal or greater efficacy was observed when some of the oils were exposed to the Gram-negative bacteria. Bergamot, cinnamon bark, cinnamon leaf, clove, grapefruit, lime, rose geranium and rosewood all produced greater zones of inhibition in some Gram-negative bacteria compared to zones produced for Gram-positive bacteria. Of the oils tested for MIC, cinnamon leaf, clove, rosewood, tea tree and thyme had lower or equal MIC for Gram-negative bacteria. Cinnamon bark EO demonstrated bactericidal effects against all the strains used in this study at very low concentrations (i.e. ≤0·25% v/v).

Cinnamon bark EO was thus selected for further investigation due to the broad-spectrum activity demonstrated against all the bacteria species tested and as it was the only oil to exert antimicrobial activity against the two strains of *P. aeruginosa* during the antimicrobial screening phase.

GC-MS analysis showed that the major component of cinnamon leaf oil used in this study was eugenol, which was 84·5% of the total oil composition. This is similar to findings in the current literature (Mallavarapu *et al.* 1995; Paranagama *et al.* 2001; Raina *et al.* 2001; Jayawardena and Smith 2010). In contrast, the major component of the cinnamon bark EO used in this study was Σ-cinnamaldehyde at 85·3% of the total composition. The high levels of this component in the bark EO has been demonstrated in other studies (Paranagama *et al.* 2001; Jayawardena and Smith 2010; Shahina *et al.* 2018). This contrasting composition of the two oils provides an explanation of their different antimicrobial effects, and it has been shown that cinnamaldehyde has a superior antimicrobial effect when compared to eugenol (López *et al.* 2007; Sanla-Ead *et al.* 2011; Brnawi *et al.* 2018). The differences seen in oil composition also highlight the

importance of adequate analysis of EOs, using methods such as GC-MS, to confirm the origin of the oil and identify which part of the plant it is derived from.

From the time-kill assay in the present study, cinnamon bark EO exhibited bactericidal effects with rapid killing action (three-log reduction) at ≤30 min at concentrations ≥0·25% (v/v). These findings are further evidence of the broad-spectrum potential of EOs, against both Gram-negative and Gram-positive bacteria. The TTC assay was used to indicate the presence or absence of metabolically active cells which would convert TTC to a coloured formazan derivative (Sabaeifard *et al.* 2014). This assay is an important indicator for bacterial viability, as the intensity of the colour measured is directly proportional to the number of living cells (Moussa *et al.* 2013). The results obtained correlate well with the time-kill assay. The assay provides evidence for the possible mode of action of cinnamon bark EO, suggesting loss of metabolic function in the *Ps. aeruginosa* strain following exposure. These findings indicate that the respiratory activity necessary to reduce TTC was not present, which could also indicate a loss of membrane-selective permeability and loss of other vital enzymatic actions, as proposed by Bouhdid *et al.* (2010). However, it has been reported that TTC reduction may not correlate exactly with MIC, and end points are less easily determined using the TTC assay when compared to that of other redox indicators, such as resazurin (Mann and Markham 1998).

The bactericidal effect of cinnamon bark EO on *P. aeruginosa* cell morphology was examined by SEM. Only after the 10 min of exposure to the EO, morphological changes to the treated cells were pronounced, and suggested total cell collapse and likely loss of viability. Reports on the mechanism of action of EOs suggest their role in the destruction of the cell wall, damage to the cytoplasmic membrane and membrane proteins, and cell leakage (Goldbeck *et al.* 2014). This suggestion is supported by data from the membrane permeability assay, which indicate that cinnamon bark EO causes leakage of nucleic acids, a probable result of a compromised cell membrane. This is in agreement with Bouhdid *et al.* (2010) who found that cinnamon bark EO affected the membrane of *P. aeruginosa* which ultimately led to cell death. This contrasts with the findings by Cox and Markham (2007) and Helander *et al.* (1998) who showed that Σ-cinnamaldehyde, a predominant component of the cinnamon bark EO used in this study, did not have an effect on the membrane of *P. aeruginosa* and was deemed to not be a pore-forming compound. This highlights the importance of using whole oils, and not their isolated compounds, as it may not reflect the antimicrobial capability of an oil. It also further highlights the need for oil analysis to identify the blend of different compounds present and the role they play in the antimicrobial effects of an EO.

Cinnamon EO is sourced from either the bark or the leaf of the plants (Park *et al.* 2018), with the EO extracted from bark most often investigated for its antimicrobial activity (Nabavi *et al.* 2015). Cinnamon bark EO is already well-described for its antimicrobial activity (Manso *et al.* 2014; Wu *et al.* 2015; Wen *et al.* 2016; Zhang *et al.* 2016; Chouhan *et al.* 2017), and shown to be highly bactericidal against *Pseudomonas* sp. (Utchariyakiat *et al.* 2016). However, this study is the first to demonstrate to our knowledge the rapid killing effect of the cinnamon bark EO against *P. aeruginosa,* with contact times of <30 min.

In summary, this study has shown that EOs are effective antimicrobial agents and many possess broad-spectrum activity. Cinnamon bark EO in particular has demonstrated strong bacteriostatic and bactericidal action against *P. aeruginosa*. This provides a sound basis for further work to explore the use of cinnamon bark EO as an alternative to conventional antimicrobial products owing to its fast-acting bactericidal properties at low concentrations.

The present study has confirmed that cinnamon bark is an antimicrobial EO which provides broad spectrum, high bactericidal activity when used at low concentrations and within a short contact time. These qualities make it an ideal candidate as an alternative to current antimicrobials, for example on surfaces in clinical and food preparation applications, in disinfection and infection control. Many studies investigating time-kill and bactericidal activities of EOs expose bacteria to longer contact times, though it is important that shorter contact times are investigated and this study highlights the importance of performing antimicrobial tests using contact times <10 min.

Findings from this study further support the reports that mode of action of cinnamon bark EO against *P. aeruginosa* is due to the effects against the membrane of the bacteria, leading to cell leakage and cell death, shown here in the results of SEM and 260/280nm absorbance assay. Although EOs are generally recognized as safe by the Food and Drugs Administration (FDA), including that of cinnamon (Maisanaba *et al.* 2017), more investigation is needed to assess cinnamon EO's application to control bacterial pathogens and subsequent interaction with humans. This study also highlights the importance of analysing these natural products before use, in order to determine their composition and to identify their key components. The composition and antimicrobial diversity observed among oils originating from different parts of the same species of plant emphasize the need for batch control and consistency when developing these oils for antimicrobial purposes.

Acknowledgements

This work was supported by Medical Wire and Equipment (MWE) and the Faculty of Health and Applied Sciences, University of the West of England, Bristol via joint funding. EOs were kindly donated by Amphora Aromatics, Bristol. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Conflict of Interest

No conflict of interest declared.

References

Adukwu, E.C., Allen, S.C.H. and Phillips, C.A. (2012) The anti-biofilm activity of lemongrass (*Cymbopogon flexuosus*) and grapefruit (*Citrus paradisi*) essential oils against five strains of *Staphylococcus aureus*. *J Appl Microbiol* **113**, 1217–1227.

Ahmed, M.N.A. (2013) *Effects of escapin intermediate products (EIP-K) on biofilms of Pseudomonas aeruginosa*. MSc Thesis, Georgia State University.

Andrews, J.M. (2007) BSAC standardized disc susceptibility testing method (version 6). *J Antimicrob Chemother* **60**, 20–41.

Bouhdid, S., Abrini, J., Amensour, M., Zhiri, A., Espuny, M.J. and Manresa, A. (2010) Functional and ultrastructural changes in *Pseudomonas aeruginosa* and *Staphylococcus aureus* cells induced by *Cinnamomum verum* essential oil. *J Appl Microbiol* **109**, 1139–1149.

Brnawi, W.I., Hettiarachchy, N.S., Horax, R., Kumar-Phillips, G., Seo, H.-S. and Marcy, J. (2018) Comparison of cinnamon essential oils from leaf and bark with respect to antimicrobial activity and sensory acceptability in strawberry shake. *J Food Sci* **83**, 475–480.

Cao, L., Si, J.Y., Liu, Y., Sun, H., Jin, W., Li, Z., Zhao, X.H. and Pan, R.Le (2009) Essential oil composition, antimicrobial and antioxidant properties of *Mosla chinensis* Maxim. *Food Chem* **115**, 801–805.

Carson, C.F., Mee, B.J. and Riley, T.V. (2002) Mechanism of action of *Melaleuca alternifolia* (tea tree) oil on *Staphylococcus aureus* determined by time-kill, lysis, leakage, and salt tolerance assays and electron microscopy. *Antimicrob Agents Chemother* **46**, 1914–1920.

Chouhan, S., Sharma, K. and Guleria, S. (2017) Antimicrobial activity of some essential oils -present status and future perspectives. *Medicines* **4**, 1–21.

Clinical and Laboratory Standards Institute (1999) *M26-A: Methods for Determining Bactericidal Activity Of Antimicrobial Agents; Approved Guideline*. Pennsylvania, USA: CLSI.

Clinical and Laboratory Standards Institute (2012) *M07–A9: Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically; Approved Standard—Ninth Edition*. Pennsylvania, USA: CLSI.

Clinical and Laboratory Standards Institute (2015) *M02–A12: Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard—Twelfth Edition*. Pennsylvania, USA: CLSI.

Cowan, M.M. (1999) Plant products as antimicrobial agents. *Clin Microbiol Rev* **12**, 564–582.

Cox, S.D. and Markham, J.L. (2007) Susceptibility and intrinsic tolerance of *Pseudomonas aeruginosa* to selected plant volatile compounds. *J Appl Microbiol* **103**, 930–936.

Cui, H., Li, W., Li, C., Vittayapadung, S. and Lin, L. (2016) Liposome containing cinnamon oil with antibacterial activity against methicillin-resistant *Staphylococcus aureus* biofilm. *Biofouling* **32**, 215–225.

Department of Health (2016) Antimicrobial resistance empirical and statistical evidence-base. Public Health England, (September), 1–63.

Doran, A.L., Morden, W.E., Dunn, K. and Edwards-Jones, V. (2009) Vapour-phase activities of essential oils against antibiotic sensitive and resistant bacteria including MRSA. *Lett Appl Microbiol* **48**, 387–392.

EUCAST (2015) *European Committee on Antimicrobial Susceptibility Testing Breakpoint Tables for Interpretation of MICs and Zone Diameters*. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 8.0, 2018. Available at: http://www.eucast.org. 0-77

Faleiro, M. (2011) The mode of antibacterial action of essential oils. In *Science Against Microbial Pathogens: Communicating Current Research and Technological Advances* ed. Méndez-Vilas, A. pp. 1143–1156. London: World Scientific Publishing Co., Pte. Ltd.

Friedman, M., Henika, P.R., Levin, C.E. and Mandrell, R.E. (2004) Antibacterial activities of plant essential oils and their components against *Escherichia coli* O157:H7 and *Salmonella enterica* in apple juice. *J Agric Food Chem* **52**, 6042–6048.

Ghabraie, M., Vu, K.D., Tata, L., Salmieri, S. and Lacroix, M. (2016) Antimicrobial effect of essential oils in combinations against five bacteria and their effect on sensorial quality of ground meat. *LWT - Food Sci Technol* **66**, 332–339.

Goldbeck, J.C., Victoria, F.N., Motta, A., Savegnago, L., Jacob, R.G., Perin, G., Lenardão, E.J. and da Silvaa, W.P. (2014) Bioactivity and morphological changes of bacterial cells after exposure to 3-(p-chlorophenyl)thio citronellal. *LWT - Food Sci Technol* **59**, 813–819.

Gyawali, R. and Ibrahim, S.A. (2014) Natural products as antimicrobial agents. *Food Control* **46**, 412–429.

Hammer, K.A., Carson, C.F. and Riley, T.V. (1999) Antimicrobial activity of essential oils and other plant extracts. *J Appl Microbiol* **86**, 985–990.

Helander, I.M., Alakomi, H.-L., Latva-Kala, K., Mattila-Sandholm, T., Pol, I., Smid, E.J., Gorris, L.G.M. and Wright, A. (1998) Characterization of the action of selected essential oil components on gram-negative bacteria. *J Agric Food Chem* **46**, 3590–3595.

Jayawardena, B. and Smith, R.M. (2010) Superheated water extraction of essential oils from *Cinnamomum zeylanicum* (L.). *Phytochem Anal* **21**, 470–472.

Kačániová, M., Terentjeva, M., Vukovic, N., Puchalski, C., Roychoudhury, S., Kunová, S., Klüga, A., Tokár, M. *et al.* (2017) The antioxidant and antimicrobial activity of essential oils against *Pseudomonas* spp. isolated from fish. *Saudi Pharmaceut J* **25**, 1108–1116.

Kavanaugh, N.L. and Ribbeck, K. (2012) Selected antimicrobial essential oils eradicate *Pseudomonas* spp. and *Staphylococcus aureus* biofilms. *Appl Environ Microbiol* **78**, 4057–4061.

Lodhia, M.H., Bhatt, K.R. and Thaker, V.S. (2009) Antibacterial activity of essential oils from palmarosa, evening primrose, lavender and tuberose. *Indian J Pharmaceut Sci* **71**, 134–136.

López, P., Sánchez, C., Batlle, R. and Nerín, C. (2007) Vapor-phase activities of cinnamon, thyme, and oregano essential oils and key constituents against foodborne microorganisms. *J Agric Food Chem* **55**, 4348–4356.

Maisanaba, S., Llana-Ruiz-Cabello, M., Gutiérrez-Praena, D., Pichardo, S., Puerto, M., Prieto, A.I., Jos, A. and Cameán, A.M. (2017) New advances in active packaging incorporated with essential oils or their main components for food preservation. *Food Rev Int* **33**, 447–515.

Mallavarapu, G.R., Ramesh, S., Chandrasekhara, R.S., Rajeswara Rao, B.R., Kaul, P.N. and Bhattacharya, A.K. (1995) Investigation of the essential oil of cinnamon leaf grown at Bangalore and Hyderabad. *Flavour Fragr J* **10**, 239–242.

Mann, C.M. and Markham, J.L. (1998) A new method for determining the minimum inhibitory concentration of essential oils. *J Appl Microbiol* **84**, 538–544.

Manso, S., Pezo, D., Gómez-Lus, R. and Nerín, C. (2014) Diminution of aflatoxin B1 production caused by an active packaging containing cinnamon essential oil. *Food Control* **45**, 101–108.

Miksusanti, M., Jenie, S.L., Priosoeryanto, B.P., Syarief, R. and Rekso, G.T. (2008) Mode of action temu kunci (*kaempferia pandurata*) essential oil on *E. coli* k1.1 cell determined by leakage of material cell and salt tolerance assays. *HAYATI J Biosci* **15**, 56–60.

Millezi, A.F., Piccoli, R.H., Oliveira, J.M. and Pereira, M.O. (2016) Anti-biofim and antibacterial effect of essential oils and their major compounds. *J Essent Oil Bear Pl* **19**, 624–631.

Moussa, S.H., Tayel, A.A., Al-Hassan, A.A. and Farouk, A. (2013) Tetrazolium/formazan test as an efficient method to determine fungal chitosan antimicrobial activity. *J Mycol* **2013**, 1–7.

Murtey, M.Das and Ramasamy, P. (2016) Sample preparations for scanning electron microscopy – life sciences. In *Modern Electron Microscopy in Physical and Life Sciences* InTech ed. Janecek, M. pp. 161–185.

Nabavi, S.F., Di Lorenzo, A., Izadi, M., Sobarzo-Sánchez, E., Daglia, M. and Nabavi, S.M. (2015) Antibacterial effects of cinnamon: from farm to food, cosmetic and pharmaceutical industries. *Nutrients* **7**, 7729–7748.

Nikaido, H. (2009) Multidrug resistance in bacteria. *Annu Rev Biochem* **78**, 119–146.

Paranagama, P.A., Wimalasena, S., Jayatilake, G.S., Jayawardena, A.L., Senanayake, U.M. and Mubarak, A.M. (2001) A comparison of essential oil constituents of bark, leaf, root and fruit of cinnamon (*Cinnamomum zeylanicum* Blume) grown in Sri Lanka. *J Natl Sci Found* **29**, 147–153.

Park, J.B., Kang, J.H. and Song, K.B. (2018) Antibacterial activities of a cinnamon essential oil with cetylpyridinium chloride emulsion against *Escherichia coli* O157:H7 and *Salmonella Typhimurium* in basil leaves. *Food Sci Biotechnol* **27**, 47–55.

Porter, N.G. and Wilkins, A.L. (1998) Chemical, physical and antimicrobial properties of essential oils of *Leptospermum scoparium* and *Kunzea ericoides*. *Phytochemistry* **50**, 407–415.

Priti, V. and Shridhar, P. (2012) Effect of essential oils on MDR pathogens: a comparative study. *J Environ Res Dev* **6**, 462–466.

Raina, V.K., Srivastava, S.K., Aggarwal, K.K., Ramesh, S. and Kumar, S. (2001) Essential oil composition of *Cinnamomum zeylanicum* Blume leaves from Little Andaman, India. *Flavour Fragr J* **16**, 374–376.

Roshan, N., Hammer, K.A. and Riley, T.V. (2018) Non-conventional antimicrobial and alternative therapies for the treatment of *Clostridium difficile* infection. *Anaerobe* **49**, 103–111.

Sabaeifard, P., Abdi-Ali, A., Soudi, M.R. and Dinarvand, R. (2014) Optimization of tetrazolium salt assay for *Pseudomonas aeruginosa* biofilm using microtiter plate method. *J Microbiol Methods* **105**, 134–140.

Sakkas, H., Gousia, P., Economou, V., Sakkas, V., Petsios, S. and Papadopoulou, C. (2016) *In vitro* antimicrobial activity of five essential oils on multidrug resistant Gram-negative clinical isolates. *J Intercult Ethnopharmacol* **5**, 212–218.

Sanla-Ead, N., Jangchud, A., Chonhenchob, V. and Suppakul, P. (2011) Antimicrobial activity of cinnamaldehyde and eugenol and their activity after incorporation into cellulose-based packaging films. *Packag Technol Sci* **25**, 7–17.

Semeniuc, C.A., Pop, C.R. and Rotar, A.M. (2017) Antibacterial activity and interactions of plant essential oil combinations against Gram-positive and Gram-negative bacteria. *J Food Drug Anal* **25**, 403–408.

Shahina, Z., El-Ganiny, A.M., Minion, J., Whiteway, M., Sultana, T. and Dahms, T.E.S. (2018) *Cinnamomum*

zeylanicum bark essential oil induces cell wall remodelling and spindle defects in *Candida albicans*. *Fungal Biol Biotechnol* **5**, 1–16.

Tangjitjaroenkun, J., Chavasiri, W., Thunyaharn, S. and Yompakdee, C. (2012) Bactericidal effects and time-kill studies of the essential oil from the fruits of *Zanthoxylum limonella* on multi-drug resistant bacteria. *J Essent Oil Res* **24**, 363–370.

Tanwar, J., Das, S., Fatima, Z. and Hameed, S. (2014) Multidrug resistance: an emerging crisis. *Interdiscip Perspect Infect Dis* **2014**, 1–7.

Tracanna, V., de Jong, A., Medema, M.H. and Kuipers, O.P. (2017) Mining prokaryotes for antimicrobial compounds: from diversity to function. *FEMS Microbiol Rev* **41**, 417–429.

Utchariyakiat, I., Surassmo, S., Jaturanpinyo, M., Khuntayaporn, P. and Chomnawang, M.T. (2016) Efficacy of cinnamon bark oil and cinnamaldehyde on anti-multidrug resistant *Pseudomonas aeruginosa* and the synergistic effects in combination with other antimicrobial agents. *BMC Complement Altern Med* **16**, 1–7.

Wen, P., Zhu, D.-H., Wu, H., Zong, M.-H., Jing, Y.-R. and Han, S.-Y. (2016) Encapsulation of cinnamon essential oil in electrospun nanofibrous film for active food packaging. *Food Control* **59**, 366–376.

Wu, J., Liu, H., Ge, S., Wang, S., Qin, Z., Chen, L., Zheng, Q., Liu, Q. *et al.* (2015) The preparation, characterization, antimicrobial stability and *in vitro* release evaluation of fish gelatin films incorporated with cinnamon essential oil nanoliposomes. *Food Hydrocolloids* **43**, 427–435.

Yap, P.S.X., Yiap, B.C., Ping, H.C. and Lim, S.H.E. (2014) Essential oils, a new horizon in combating bacterial antibiotic resistance. *Open Microbiol J* **8**, 6–14.

Zhang, Y., Liu, X., Wang, Y., Jiang, P. and Quek, S. (2016) Antibacterial activity and mechanism of cinnamon essential oil against *Escherichia coli* and *Staphylococcus aureus*. *Food Control* **59**, 282–289.