

# ARE ESSENTIAL OILS A SOLUTION TO COMBAT ANTIMICROBIAL RESISTANCE?

---



UNIVERSITY OF THE  
WITWATERSRAND,  
JOHANNESBURG

**Keruné Naidoo**

A dissertation by publication submitted to the Faculty of Health Sciences, University of the  
Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of  
Pharmacy

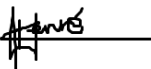
**Johannesburg, 2024**

# DECLARATION: Student's contribution to the article and agreement of co-authors

---

---

I, Keruné Naidoo, student number 1598125, declare that this Dissertation is my own work and that I contributed in full towards the research findings published in the article stated below which are included in my Dissertation.

Signature of Student 




Date 22 July 2024

Agreement by co-authors: By signing this declaration, the co-authors listed herein agree to the use of the article by the student as part of her Dissertation.

**Article Title:** Unveiling the Potential of Essential Oils against Bacterial Resistance

**Journal submitted to:** Journal of Essential Oil Bearing Plants, Taylor & Francis, on the 22<sup>nd</sup> of July 2024.

**Contribution per author:** **A. Orchard and S. van Vuuren:** Conceptualization, resources, review and editing, supervision, project administration, funding acquisition. **K. Naidoo:** Methodology, formal analysis and laboratory experimental work, data curation, writing-original draft preparation and review and editing. All authors reviewed the drafts and contributed towards editing before the submission for publication.

| Authors  | Name             | Signature  | Date         |
|----------|------------------|--|--------------|
| Author 1 | Keruné Naidoo    |   | 22 July 2024 |
| Author 2 | Sandy van Vuuren |   | 22 July 2024 |
| Author 3 | Ané Orchard      |  | 22 July 2024 |

# DEDICATION

---

---

To my best friend, Rosebud Naidoo, no amount of words could sum up the impact you have had on my life and the unconditional love you have given me over the years. You were the greatest companion in every way possible, and your presence alone got me through the toughest of days. Your memory will live on in my heart forever. – April 2009 - March 2024

To my parents, who have always believed in me and constantly showered me with unconditional love. This achievement would not have been possible without you. You always strive to give me the best of everything, and I am eternally grateful for your unwavering support. Thank you, Mum and Pa, for all that you do. I am incredibly lucky to have you both as my parents.

*This dissertation is dedicated to you, my pillars of strength.*

# LIST OF PRESENTATIONS AND PUBLICATION ARISING FROM THIS STUDY

---

---

## **Presentations**

K Naidoo, A Orchard, SF van Vuuren. Are essential oils a solution to combat antimicrobial resistance? Presented at the following meetings: 1. School of Therapeutic Sciences Research Day (STHS) 2023, 5 September 2023, Johannesburg, University of the Witwatersrand. [oral presentation] (APPENDIX A1); 2. South African Association of Botanists (SAAB) Postgraduate Symposium 2023, 28 September 2023, Virtual. [oral presentation] (APPENDIX A2).

K Naidoo, A Orchard, SF van Vuuren. The antimicrobial potential of essential oils against the ESKAPE pathogens. South Africa. Molecular Biosciences Research Thrust (MBRT) 2023, 7 December 2023, Johannesburg, University of the Witwatersrand. [oral presentation] (APPENDIX A3)

## **Publication (Chapter 3)**

Naidoo, K., Orchard, A., and van Vuuren, S.F., Unveiling the Potential of Essential Oils against Bacterial Resistance. Submitted to Journal of Essential Oil Bearing Plants (July 2024).

## ABSTRACT

---

---

Essential oils (EOs), derived from aromatic plants, have been widely studied and used as antimicrobials due to their broad-spectrum activity. This offers a potential resolution to the worldwide concern of antimicrobial resistance. However, little is known about the impact EOs will have on resistance emergence due to exposure in relation to resistance mechanisms. This study aimed to investigate resistance mechanisms and the potential induction of resistance against a selection of EOs. The research objectives encompassed four methods comprising of an initial antimicrobial screening of 23 EOs, followed by anti-quorum sensing (anti-QS) activity, biofilm inhibition, and resistance induction assays against five oils.

The broth microdilution assay was carried out on 23 EOs, to screen the minimum inhibitory concentrations (MICs) against the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter aerogenes*) pathogens. This included reference, susceptible and resistant clinical strains. Based on the minimum inhibitory activity results, five EOs, *Thymus vulgaris* Willk. (0.92 mg/mL), *Origanum vulgare* L. (0.72 mg/mL), *Carum carvi* L. (1.62 mg/mL), *Commiphora myrrha* Engl. (3.04 mg/mL), and *Matricaria recutita* L. (3.36 mg/mL), representing a variety of inhibitory activity (noteworthy - poor) were selected for further investigation into resistance mechanisms.

The anti-QS activity was evaluated, using *Chromobacterium violaceum* (ATCC 12472) as the biomonitor strain. The results revealed that all five EOs exhibited noteworthy ( $\geq 70.00\%$ ) quorum sensing (QS) inhibition at concentrations ranging from 0.02 - 1.00 mg/mL. Essential oils with noteworthy and moderate inhibitory activities had comparable minimum quorum sensing inhibitory concentrations (MQSICs). For instance, *C. carvi* and *O. vulgare* both exhibited a MQSIC ranging from 0.03 to 0.06 mg/mL; though, *O. vulgare* displayed a 3.01% higher inhibition of QS compared to *C. carvi*.

For the biofilm inhibition assay, the crystal violet method was used to quantify the biofilm inhibition percentage. The five EOs were tested against *S. aureus* ATCC 6538. At a concentration of 0.05 mg/mL, *O. vulgare* and *T. vulgaris* inhibited biofilm formation most notably ( $\geq 70.00\%$ ). *Matricaria recutita* and *C. myrrha* inhibited more than 70.00% biofilm

formation starting at 2.00 mg/mL, while *C. carvi* only demonstrated notable inhibition ( $\geq 70.00\%$ ) at the highest concentration (4.00 mg/mL) tested.

The resistance induction assay involved exposure of *S. aureus* ATCC 6538, to the five EOs, over a total of 20 passages. *Origanum vulgare* and *T. vulgaris* maintained their noteworthy minimum inhibitory activity over the 20 passages with minor inconsistencies in the MIC (SD  $\pm 0.22$  and  $\pm 0.32$  respectively). *Carum carvi* had the most variation in the MIC reported, however, no occurrences of resistance were detected. *Commiphora myrrha* and *M. recutita* both had a sudden declivity in their MIC at passage 16, with the MIC difference being more than two serial dilutions lower. There were changes in the growth kinetics of *S. aureus* as a result of exposure, which was observed in passages 16, 17, and 18. The exponential phase, when resistance is most likely to occur, shifted from 3 to 11 hrs, between passages 16 and 17. *Staphylococcus aureus* ATCC 6538 displayed heteroresistance patterns to the antibiotics after the exposure of the culture to EOs at certain passages. The exposure of *S. aureus* to *O. vulgare* resulted in the most variation in the MIC of the antibiotics, with *T. vulgaris* being the second.

This study revealed that *O. vulgare* and *T. vulgaris* exhibited noteworthy activity in the minimum inhibition, anti-QS, and biofilm inhibition assays, within a concentration range of 0.03 - 1.00 mg/mL and 0.02 - 1.00 mg/mL respectively, highlighting these oils as promising antimicrobials to combat resistance. The induction of resistance assay gave key insight into the relationship between minimum inhibitory activity and the ability to detect resistance through repeated exposure. In its entirety, the study affirms the dissertation title by demonstrating that EOs can effectively contribute to combating AMR, contingent upon meticulous selection and a thorough understanding of the role each EO plays in various resistance mechanisms.

## ACKNOWLEDGEMENTS

---

---

To my supervisors Professor Sandy van Vuuren and Doctor Ané Orchard, I am thankful for your continuous guidance and compassion throughout this journey, particularly during the challenging times towards the end. I truly appreciate the chance to pursue my passion under your supervision, igniting a stronger desire to expand my knowledge through research. Thank you, Prof, for your consistent concern about my well-being. Thank you, Doctor Orchard, for all your pep talks, which helped me through my anxiety.

*'Matha, Pitha, Guru, Daivam,'* translates to 'Mother, Father, Teacher, God', highlighting the important role that each has in the growth of the individual. Thank you, Dear Bhagawan Sri Sathya Sai Baba, He who is omniscient, omnipresent, and omnipotent, for providing me with guidance and offering calmness during times of adversity through your teachings, always.

To my loving boyfriend, Reon Naidoo, you bring so much joy and light into my life. Thank you for being my rock and my source of endless love and support. Thank you for standing by my side from the very start, taking me to campus at all odd hours of the night to remove my culture from the incubators, reading my results with me and uplifting me during moments of doubt and discouragement. Thank you for listening and trying to understand all the assays I was doing, so I could explain the problems I had in the laboratory with you. You are one in eight billion.

To the best brother, Yaashlin Naidoo, life would be so boring and glum without you, no matter what I am going through or how bad my day was, you always make me laugh and make me feel better. Thank you for always sharing your great insights with me, for my betterment. I know I can always depend on you. Having a brother like you is one of life's greatest blessings.

To my fur baby, Qaira Naidoo, thank you for the immense joy, love and entertainment you bring to our family, you are truly one of a kind.

To my fellow master's colleague, Mr Shivar Simbu, “my third supervisor”, I express my sincere gratitude for your wealth of knowledge and invaluable advice throughout this journey. Your support has been instrumental in reaching this significant milestone, and I am truly grateful to

have done my master's alongside such a dedicated individual like you. Thank you for consistently engaging in brainstorming sessions with me and for sharing relevant research papers that greatly benefited my work.

To Phumzile Moerane, our lab technician, thank you for your consistent support and guidance. I deeply appreciate your invaluable contributions to the smooth operation of the laboratory, which allowed me to carry out my work optimally. Thank you for answering my calls even after hours when I desperately needed to know where something was.

To Tama Mwale and Muneebah Laher, my fellow interns, thank you for your company and understanding through the most difficult year, I thoroughly enjoyed learning new methods with you, it made the whole process that more exciting. Thank you sincerely, for your continuous words of encouragement.

To my family, thank you for your love, laughter, support, and encouragement throughout my master's journey. Thank you for being there to lend a listening ear, and simply inviting me over for a meal, it was a much-needed break to rejuvenate before returning to the laboratory.

To Dr. Teena Thomas from NHLS Infection Control and Microbiology Laboratory, thank you for generously supplying the clinical strains used in this study.

The Faculty Research Committee (FRC) is thanked for financial assistance.

National Research Foundation (NRF) is thanked for the Thuthuka grant funding (129672), on behalf of Dr Orchard.

The University of Witwatersrand is thanked for the facilities and equipment provided for my research.

# DISSERTATION STRUCTURE

---

A dissertation by publication was undertaken and comprises of the following;

- Chapter one presents the study and elucidates its design.
- Chapter two presents a review of the literature.
- Chapter three presents the publication titled " Unveiling the Potential of Essential Oils against Bacterial Resistance".
- Chapter four provides an overview and concludes the study.

# TABLE OF CONTENTS

---

---

|   |      |
|---|------|
| DECLARATION: Student's contribution to the article and agreement of co-authors .....        | i    |
| DEDICATION .....  | ii   |
| LIST OF PRESENTATIONS AND PUBLICATION ARISING FROM THIS STUDY .....                         | iii  |
| ABSTRACT.....   | iv   |
| ACKNOWLEDGEMENTS.....   | vi   |
| DISSERTATION STRUCTURE.....   | viii |
| TABLE OF CONTENTS.....  | ix   |
| LIST OF FIGURES .....   | xi   |
| LIST OF TABLES .....  | xii  |
| LIST OF EQUATIONS.....  | xiii |
| LIST OF ABBREVIATIONS .....   | xv   |
| CHAPTER 1: INTRODUCTION .....   | 1    |
| 1.1 Background.....   | 1    |
| 1.2 Essential oils .....  | 1    |
| 1.3 Antimicrobial resistance.....   | 2    |
| 1.3.2 <i>ESKAPE pathogens</i> .....   | 3    |
| 1.3.3 <i>Quorum sensing</i> .....   | 3    |
| 1.3.4 <i>Planktonic versus sessile inhibition</i> .....                                     | 4    |
| 1.3.5 <i>Sub-inhibitory exposure to essential oils</i> .....                                | 4    |
| 1.4 Problem statement.....  | 5    |
| 1.5 Essential oils selected .....   | 5    |
| 1.6 Aims and objectives .....   | 8    |
| CHAPTER 2: LITERATURE REVIEW .....  | 9    |
| 2.1 The planktonic inhibitory effect of essential oils on the <i>ESKAPE pathogens</i> ..... | 9    |
| 2.2 Essential oil's ability to overcome the emergence of bacterial resistance .....         | 16   |
| 2.2.1 <i>Anti-quorum sensing</i> .....  | 17   |
| 2.2.1.1 <i>The role of quorum sensing on pathogenicity</i> .....                            | 17   |

|  |     |
|--|-----|
| 2.2.1.2 <i>Anti-quorum sensing activity of essential oils</i> .....                                      | 19  |
| 2.2.2 <i>Biofilm inhibition</i> .....  | 20  |
| 2.2.2.1 <i>The role of biofilm inhibition on pathogenicity</i> .....                                     | 20  |
| 2.2.2.2 <i>Biofilm inhibitory potential of essential oils</i> .....                                      | 21  |
| 2.2.3 <i>Induction of resistance</i> .....   | 21  |
| 2.2.3.1 <i>Mechanisms bacteria use to induce resistance</i> .....  | 21  |
| 2.2.3.2 <i>Essential oils and induction of bacterial resistance</i> .....                                | 28  |
| CHAPTER 3: PUBLICATION .....   | 32  |
| CHAPTER 4: OVERVIEW AND CONCLUSION .....   | 94  |
| 4.1. Study highlights .....  | 94  |
| 4.2. Integration of all four assays .....  | 94  |
| 4.2.1 <i>Minimum inhibitory activity and anti-quorum sensing activity</i> .....                          | 95  |
| 4.2.2 <i>Minimum inhibitory activity and biofilm inhibition</i> .....                                    | 96  |
| 4.2.3 <i>Anti-quorum sensing and biofilm inhibition</i> .....  | 97  |
| 4.2.4 <i>Minimum inhibitory activity and induction of resistance</i> .....                               | 98  |
| 4.3 Future recommendations regarding antimicrobial resistance studies in essential oil<br>research ..... | 98  |
| 4.4 Final remarks .....  | 101 |
| REFERENCES .....   | 103 |
| APPENDIX A1 - Abstract for presentation (STHS).....  | 125 |
| APPENDIX A2 - Abstract for presentation (SAAB).....  | 126 |
| APPENDIX A3 - Abstract for presentation (MBRT) .....   | 127 |
| APPENDIX B - Ethics waiver .....   | 128 |
| APPENDIX C - Plagiarism declaration .....  | 129 |
| APPENDIX D - Turnitin report .....   | 130 |
| APPENDIX E - Author permission .....   | 131 |

# LIST OF FIGURES

---

---

|   |    |
|---|----|
| <b>Figure 2.1.</b> Quorum sensing pathways leading to resistance development in the ESKAPE pathogens (Ghosh et al.2022).....  | 19 |
| <b>Figure 2.2.</b> The emergence of resistance after the introduction of the antibiotic from 1935 to 2017 (Revised from Stennett et al. (2022) and Helmy et al.(2023)).....                               | 32 |
| <b>Figure 4.1.</b> The comparison of consistency of the antimicrobial activity between the EOs and the three conventional antibiotics (ciprofloxacin, erythromycin, and gentamicin) over 20 passages..... | 40 |

## LIST OF TABLES

---

---

|  |    |
|--|----|
| <b>Table 1.5.</b> Essential oil selection for this study is based on reported antimicrobial activity against the ESKAPE pathogens..... | 5  |
| <b>Table 2.1.</b> Minimum inhibitory concentrations of 23 essential oils against the ESKAPE pathogens.....                             | 10 |
| <b>Table 2.2.</b> Anti-quorum sensing studies on the 23 essential oils investigated in this study.....                                 | 21 |
| <b>Table 2.3.</b> Known biofilm inhibition studies on essential oils from this study.....  | 28 |

# LIST OF EQUATIONS

---

---

## Equation 1:

$$\text{Percentage violacein production} = \frac{\text{control Optical density (OD)} - \text{OD experimental}}{\text{control OD}} \times 100$$

## Equation 2:

$$\text{Percentage biofilm inhibition} = \frac{\text{control Optical density (OD)} - \text{OD experimental}}{\text{control OD}} \times 100$$

## LIST OF ABBREVIATIONS

---

---

|                    |   |
|--------------------|---|
| $\alpha$           | Alpha   |
| $\beta$            | Beta  |
| $\gamma$           | Gamma   |
| $\mu\text{g}$      | Microgram   |
| $\mu\text{L}$      | Microlitre  |
| $\mu\text{l/L}$    | Microlitre per litre                              |
| $\mu\text{l/mL}$   | Microlitre per millilitre                         |
| $^{\circ}\text{C}$ | Degrees celsius                                   |
| %                  | Percentage  |
| <                  | Less than   |
| >                  | Greater than                                      |
| $\leq$             | Less than or equal to                             |
| $\geq$             | Greater than or equal to                          |
| AMR                | Antimicrobial resistance                          |
| ATCC               | American type culture collection                  |
| EO                 | Essential oil                                     |
| CFU                | Colony forming units                              |
| DMSO               | Dimethyl sulfoxide                                |
| g                  | Gram  |
| hrs                | Hours   |
| INT                | <i>p</i> -iodonitrotetrazolium violet             |
| L                  | Litre   |
| LB                 | Luria Bertani                                     |
| MAR                | Multiple antibiotic resistant                     |
| mg                 | Milligram   |
| mg/L               | Milligram per litre                               |
| mg/mL              | Milligram per millilitre                          |
| MIC                | Minimum inhibitory concentration                  |
| min                | Minutes   |
| mL                 | Millilitre  |
| MQSBC              | Minimum quorum sensing bactericidal concentration |

|       |  |
|-------|--|
| MQSIC | Minimum quorum sensing inhibitory concentration    |
| MRSA  | Methicillin-resistant <i>Staphylococcus aureus</i> |
| NCTC  | National collection of type cultures               |
| NHLS  | National Health Laboratory Service                 |
| QS    | Quorum sensing                                     |
| sec   | Seconds  |
| SD    | Standard deviation                                 |
| TSA   | Tryptone Soya agar                                 |
| TSB   | Tryptone Soya broth                                |
| v/v   | Volume per volume                                  |
| WHO   | World Health Organisation                          |
| w/v   | Weight per volume                                  |

# CHAPTER 1: INTRODUCTION

---

---

## 1.1 Background

Antimicrobial resistance (AMR) presents a substantial challenge to global health as it decreases the effectiveness of antibiotics and other antimicrobial agents that have traditionally been successful in treating various types of infections caused by bacteria, viruses, fungi, and parasites (Murray et al., 2022). This problem is exacerbated by the overuse and inappropriate use of antimicrobials in human medicine, agriculture, and veterinary practices, leading to the emergence of multi-drug resistant (MDR) strains and prolonged illnesses with higher mortality rates. The World Health Organization (WHO) has estimated that around 4.95 million deaths every year are linked to AMR (WHO, 2023). A study in 2022 by the Lancet discovered that in 2019, 1.27 million deaths occurred due to drug-resistant bacterial infections, with 860,000 of these deaths occurring in Africa (Murray et al., 2022). Studies are also showing that the COVID-19 pandemic increased the use of antimicrobials considerably, which potentiated resistance against antibiotics (Sokolović et al. 2022, Avershina et al. 2023, Massarine et al. 2023). In fact, Taleb et al. (2023) compared the AMR profiles of bacterial pathogens pre- and post-COVID and found that there was a notable increase in resistance to several antibiotics (cephalexin, co-trimoxazole, erythromycin, cloxacillin and amoxicillin/clavulanic acid).

Neglecting the challenge of AMR not only puts individual patients at risk but also poses a threat to public health by diminishing the ability to control infectious diseases. Addressing this issue necessitates a comprehensive approach that emphasizes the judicious use of antimicrobials, global collaboration in surveillance and regulation, and the exploration of innovative alternatives to conventional antibiotics (Yap et al. 2014). An exceedingly popular natural alternative approach is the use of essential oils (EOs).

## 1.2 Essential oils

Essential oils are complex mixtures of volatile compounds distilled from various parts of aromatic plants, including flowers, leaves, stems, roots, and fruits (Aelenei et al. 2016). These oils are characterized by their intense aroma and are primarily comprised of terpenes, phenolics, and other organic compounds. Essential oils are composed of hundreds of

compounds, each with its unique biological properties, thereby exhibiting a variety of pharmacological effects (Jini 2023). The use of these oils can be traced back to ancient civilizations, where they were highly valued for their therapeutic benefits (Elshafie and Camele 2017). In recent years, there has been a significant increase in the utilization of EOs across various fields such as medicine, aromatherapy, and cosmetology (Naeem et al. 2018). Essential oils exhibit a diverse array of therapeutic effects and have been utilized in the treatment of conditions including cancer, respiratory tract disorders, cardiovascular ailments, obesity, and diabetes (Chouhan et al. 2017). One particularly valuable characteristic of EOs, that has become increasingly appealing, is their broad-spectrum antimicrobial activity against MDR pathogens (Nazzaro et al. 2013, Patterson et al. 2019).

### **1.3 Antimicrobial resistance**

Antimicrobial resistance is defined as the ability of micro-organisms to acquire resistance to counteract the effects of antibiotics. This resistance is attributed to genetic alterations, alterations to the antimicrobial molecule, hindering antimicrobial compounds from reaching their target, modifying or bypassing target sites, and resistance caused by overall cellular adaptation processes in the micro-organisms, resulting in lowered or complete ineffectiveness of these antibiotics (Munita and Arias 2016). These genetic alterations may occur naturally over time, however, the improper and excessive use of antimicrobial agents hastened the development of resistance. The emergence of AMR presents a substantial challenge to the medical field as it impedes the efficacy of antibiotics in treating infections caused by resistant pathogens (WHO 2023, Sartorius et al. 2024).

The era of antibiotics commenced with the discovery of penicillin by Sir Alexander Fleming in 1928 (Pidcock 2012). Since then, antibiotics have had a transformative impact on modern medicine and have been responsible for saving countless lives (Gould and Bal 2013). The prescribing of antibiotics for the treatment of severe infections began in the 1940s (Sengupta et al. 2013). However, the emergence of resistance to penicillin soon became a significant clinical challenge, thereby jeopardizing the progress made in the previous decade (Ventola 2015). In response, new beta-lactam antibiotics were found, developed, and utilized, which led to the restoration of confidence against infectious diseases (Spellberg and Gilbert 2014, Ventola 2015). Nonetheless, the first instance of methicillin-resistant *Staphylococcus aureus* (MRSA) was identified during the same decade, with occurrences in the United

Kingdom in 1962 and the United States in 1968 (Sengupta et al. 2013). Unfortunately, soon after an antibiotic was introduced, resistance was detected (Ventola 2015). The pharmaceutical industry developed many new antibiotics between the late 1960s through the early 1980s to curb the problem of resistance, however, after this period the rate of AMR emergence increased, and fewer new drugs were able to keep up with increasing resistance patterns (Spellberg and Gilbert 2014).

### **1.3.2 ESKAPE pathogens**

The acronym "ESKAPE" refers to a group of bacterial pathogens that have developed significant resistance to antimicrobial agents and are well-known for "escaping" the effects of conventional treatments (Panda et al. 2022). These pathogens include *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species (Ma et al. 2020). They are accountable for a diverse range of hospital-acquired infections, such as systemic infections, pneumonia, post-surgical infections and urinary tract infections. It is imperative to prioritize efforts in combating ESKAPE pathogens due to their capacity to cause severe illnesses, their inclination to spread in healthcare environments, and the limited treatment options available owing to their multi-drug resistance profiles (Mulani et al. 2019, Li et al. 2021). Addressing the challenge posed by ESKAPE pathogens requires the development of novel antimicrobial agents, that can withstand resistance development (Panda et al. 2022).

### **1.3.3 Quorum sensing**

Bacterial communication, also known as quorum sensing (QS), is a cell-to-cell communication system that plays a vital role in regulating numerous microbial mechanisms and virulence factors (Taga and Bassler 2003). This complex communication system involves the production and detection of signalling molecules called auto-inducers (Zhao et al. 2020). These auto-inducers allow bacteria to monitor the presence and density of other bacteria in their environment, leading to co-ordinated responses among bacterial populations. This communication system not only affects bacterial behaviours such as biofilm formation, antibiotic resistance, and virulence expression, but it also contributes to the emergence of multi-drug resistance in the ESKAPE pathogens (Taga and Bassler 2003).

#### ***1.3.4 Planktonic versus sessile inhibition***

Planktonic and sessile bacterial growth represent the two different modes in which bacteria proliferate. Planktonic growth takes place when bacteria are suspended in a liquid medium, whereas sessile growth occurs when bacteria adhere to surfaces and create biofilms. Both methods of proliferation can result in the emergence of resistance to antibiotics (Al-Ouqaili 2018, Hjort et al. 2022). It is crucial to examine both planktonic and sessile bacterial growth when investigating new antimicrobials because biofilms have been observed to exhibit higher levels of antibiotic resistance compared to planktonic bacteria (Jafri et al. 2019). Biofilms offer a protective layer for bacteria, enabling them to circumvent the effects of antibiotics, thus leading to treatment failures (Rodríguez-Lázaro et al. 2018). Through comprehensive investigation into both forms of growth, researchers can enhance their understanding of antibiotic resistance mechanisms and devise more effective approaches for addressing infections associated with biofilms (Vázquez-Sánchez et al. 2015, Pekmezovic et al. 2016).

#### ***1.3.5 Sub-inhibitory exposure to essential oils***

Another area that is of importance when investigating new antimicrobials, such as EOs is understanding how exposure to sub-inhibitory concentrations of these EOs could affect resistance development (Benkwitz-Bedford et al. 2021). Studies have shown that exposure to sub-inhibitory minimum inhibitory concentrations (MICs) of antibiotics can result in the expression of resistance genes and the survival of resistant bacteria through the process of natural selection (Chakraborty et al. 2022, Sanchez-Cid et al. 2022). This is significant because it demonstrates that even low concentrations of antibiotics in the environment can contribute to the spread of resistance, potentially leading to greater levels of resistance. Furthermore, sub-inhibitory concentrations of antibiotics can induce physiological effects in bacteria, such as biofilm formation, which can enhance their ability to survive and persist (Andersson et al. 2019). Thus, in addition to the urgent requirement for alternative antimicrobial agents to curb the AMR crisis, it is crucial to assess how repeated exposure to sub-inhibitory concentrations of EOs impacts the development of resistance. For further consideration, currently, EOs are commonly incorporated at low concentrations into numerous skincare and household products that form a part of individuals' daily routines, leading to repeated exposure of sub-inhibitory concentrations to skin micro-organisms.

## 1.4 Problem statement

Antimicrobial resistance poses a growing global concern, impacting millions of individuals annually. Essential oils have been recognized as a natural alternative that offers potential solutions due to their broad-spectrum antimicrobial properties. However, there is limited research exploring the impact of EOs in inducing resistance within the medical field. Furthermore, little investigation has been done to connect EOs with varying inhibitory activity and resistance mechanisms, such as QS and biofilm formation, as well as induction of resistance. Therefore, this study aimed to elucidate the impact of EOs on AMR by highlighting relationships between inhibition, anti-QS activity, biofilm inhibition, and the induction of resistance. This investigation contributes to a more comprehensive understanding of EOs' potential as a viable solution in combating AMR. Moreover, it can contribute to the development of strategies and steer forthcoming research in the AMR area, centring on EOs.

## 1.5 Essential oils selected

For this study, 23 EOs were selected primarily based on the antimicrobial activities previously reported (Orchard et al. 2017). These EOs were selected for having noteworthy inhibitory activity against one or more of the ESKAPE pathogens. The GC-MS main constituents and suppliers for each EO are specified in Table 1.5.

**Table 1.5.** Essential oil selection for this study is based on reported antimicrobial activity against the ESKAPE pathogens.

| Essential oil                         | Common name | GC-MS main constituents  | Supplier    |
|---------------------------------------|-------------|--|-------------|
| <i>Allium sativum</i> L.              | Garlic      | Diallyl sulfide 57.10%<br>Diallyl trisulfide 19.50%                                  | Escentia    |
| <i>Carum carvi</i> L.                 | Caraway     | Carvone 64.10%<br>Limonene 34.00%  | Prana Monde |
| <i>Cinnamomum verum</i> J.Presl       | Cinnamon    | E-cinnamaldehyde 56.97%<br>Eugenyl acetate 8.96%<br>Linalool 3.17%<br>Limonene 2.80% | Prana Monde |
| <i>Cinnamomum zeylanicum</i><br>Blume | Cinnamon    | Eugenol 71.98%<br>Eugenyl acetate 4.70%  | Scatters    |

| Essential oil                            | Common name      | GC-MS main constituents   | Supplier    |
|--|------------------|---|-------------|
|  |                  | $\beta$ -caryophyllene 3.31%  |             |
| <i>Commiphora myrrha</i> Engl.           | Myrrh            | Furanooudesma-1,3 diene 46.38%<br>Curzerene 21.99%<br>Lindestrene 4.99%<br>Acetyl-8,12-epoxygermacra-1,4-7-11-tetraene 4.61%                                    | Prana Monde |
| <i>Cymbopogon citratus</i> Stapf         | Lemongrass       | Geraniol 36.99%<br>Neral 29.67%<br>Acetate de geranyl 7.48%<br>Geraniol 7.31%   | Prana Monde |
| <i>Cymbopogon martinii</i> Stapf         | Palmarosa        | Geraniol 77.80%<br>Acetate de deranyle 9.60%<br>Linalol 3.02%<br>$\beta$ -caryophyllene 2.13%   | Prana Monde |
| <i>Ferula galbaniflua</i> Boiss. & Buhse | Galbanum         | $\beta$ -Pinene 54.30%  | Prana Monde |
| <i>Foeniculum dulce</i> Mill.            | Fennel           | ( <i>E</i> )-anethole 82%<br>$\beta$ -caryophyllene 12.4%<br>1,8 cineole 19.4%  | Prana Monde |
| <i>Laurus nobilis</i> L.                 | Bay              | 1,8-cineole 54.10%<br>Acetate de terphenyl 7.50%<br>Sabinene 5.45%<br>$\alpha$ -pinene 5.32%  | Prana Monde |
| <i>Lavandula angustifolia</i> Bubani     | Lavender         | Acetate de linalyl 38.14%<br>Linalol 32.99%<br>$\beta$ -caryophyllene 3.90%<br>Cis- $\beta$ ocimene 2.99%<br>Trans- $\beta$ ocinnene 2.50%<br>1,8 cineole 0.80% | Prana Monde |
| <i>Litsea cubeba</i> Pers.               | May chang        | Geraniol 34.10%<br>Neral 32.79%<br>Limonene 14.27%  | Scatters    |
| <i>Matricaria recutita</i> L.            | German chamomile | e-trans- $\beta$ -farnesene 45.73%<br>$\alpha$ -bisabolol 9.56%   | Prana Monde |

| Essential oil  | Common name   | GC-MS main constituents  | Supplier          |
|--|---------------|--|-------------------|
|  |               | Oxyde D' $\alpha$ -bisabolol 5.22%   |                   |
| <i>Melaleuca alternifolia</i> Cheel                  | Tea tree      | Terpinen-4-ol 44.60%<br>$\gamma$ -Terpinene 16.60%<br>p-Cymene 9.60%   | Locally distilled |
| <i>Ocinum tenuiflorum</i> L.                         | Holy basil    | Eugenol 30.2%<br>$\beta$ -caryophyllene 27.9%<br>$\beta$ -elemene 18.7%  | Subtle Energies   |
| <i>Origanum vulgare</i> L.                           | Oregano       | Carvacrol 62.73%<br>$\delta$ -terpinene 9.89%<br>Thymol 5.36%<br>p-cymene 4.97%                                    | Prana Monde       |
| <i>Pelargonium graveolens</i><br>L'Hér               | Rose geranium | Citronellol 27.76%<br>Geraniol 14.09%<br>Citronellyl formate 10.32%<br>Isomethane 7.82%<br>Geranyl formate 4.66%   | Scatters          |
| <i>Pogostemon patchouli</i> Benth.                   | Patchouli     | Patchouli alcohol 30.80%<br>Delta-guai-ene 18.04%<br>$\alpha$ -guai-ene 14.98%<br>$\alpha$ -patchoulene 7.42%      | Prana Monde       |
| <i>Rosa damascena</i> Mill.                          | Rose otto     | Citronellol 34.06%<br>Geraniol 14.29%<br>Nonadecane C19 8.28%<br>Nerol 6.72%                                       | Prana Monde       |
| <i>Santalum austrocaledonicum</i>                    | Sandalwood    | 2- $\alpha$ trans bergamotol 42.26%<br>z- $\beta$ santalol 17.21%<br>Lanceol 10.08%<br>Epi- $\beta$ santalol 3.52% | Prana Monde       |
| <i>Syzygium aromaticum</i> (L.)<br>Merr. & L.M.Perry | Clove         | Eugenol 70.67%<br>Eugenol acetate 19.21%<br>Caryophyllene 8.13%  | Scatters          |
| <i>Thymus vulgaris</i> Willk.                        | Thyme         | Thymol 46.61%<br>p-cymene 17.38%<br>$\gamma$ -terpinene 9.58%  | Prana Monde       |

| Essential oil                      | Common name | GC-MS main constituents  | Supplier    |
|------------------------------------|-------------|--------------------------|-------------|
|                                    |             | Carvacrol 5.16%          |             |
|                                    |             | Linalol 4.36%            |             |
| <i>Vetiveria zizanioides</i> Stapf | Vetiver     | Acide khusenique 15.87%  | Prana Monde |
|                                    |             | Khusimol 7.93%           |             |
|                                    |             | Isovalencenol 7.39%      |             |
|                                    |             | $\alpha$ -vetivone 3.73% |             |
|                                    |             | $\beta$ -vetivone 2.67%  |             |

## 1.6 Aims and objectives

This study aimed to investigate how micro-organisms respond to a selection of EOs, and to further investigate what impact these EOs have on inducing resistance. The specific objectives for this study include;

1. To determine the minimum inhibitory activity of 23 EOs against reference, resistant and susceptible clinical strains of ESKAPE pathogens using the broth microdilution method.
2. To determine the anti-QS activity of five EOs using the broth macrodilution method.
3. To determine the biofilm inhibitory activity of five EOs against *S. aureus* ATCC 6538, using the microtiter plate method.
4. To investigate whether exposure to sub-inhibitory concentrations of five EOs induces resistance in *S. aureus* ATCC 6538.
5. To compare the minimum inhibitory activity of EOs against conventional antibiotics, after repeated exposure to the EO.

## CHAPTER 2: LITERATURE REVIEW

---

---

### 2.1 The planktonic inhibitory effect of essential oils on the ESKAPE pathogens

A review conducted by Ghosh et al. (2022) provides an in-depth overview of the calamitous nature and the crucial importance of directing attention towards the ESKAPE pathogens. Given the emphasis placed on these ESKAPE pathogens by the WHO, it is vital to supplement existing research into the potential of EOs against this group of pathogens. Through reviewing the literature, it was evident that minimum inhibitory studies of EOs against *E. faecium* and *E. aerogenes* are scarce, making the addition of these two pathogens necessary to existing literature (Negreiros et al. 2016, Zhan et al. 2022). Thus, of the many EOs investigated for antimicrobial activity against one or more of the ESKAPE pathogens, 23 have been selected for this study. Studies investigating the minimum inhibitory activity of EOs are evident, particularly when considering antimicrobial-resistant bacteria (Sakkas et al. 2016, Orchard et al. 2017, Bučková et al. 2018, Orchard et al. 2018). However, there is a significantly smaller body of literature on EOs specifically targeting the ESKAPE pathogens compared to the broader research on EOs' antimicrobial properties in general (Yu et al. 2020, Panda et al. 2022, Roman et al. 2023).

Table 2.1 summarizes the reviewed literature around the 23 EOs selected for this study. There are several assays employed to quantify inhibition, namely disc diffusion, well diffusion, broth or agar dilution. Only studies utilizing the broth microdilution assay were reviewed in Table 2.1, as it is the most standardized method, to accurately reflect the inhibitory effect of an antimicrobial on bacterial strains (Kowalska-Krochmal and Dudek-Wicher, 2021). This also enables a more precise comparison of MICs (inhibitory activity) between existing literature and the findings that will come from this study.

The EOs identified across studies to have the lowest MIC values against one or more of the ESKAPE pathogens were *Allium sativum*, *Cinnamomum verum*, *Foeniculum dulce*, *Melaleuca alternifolia*, *Origanum vulgare*, *Syzygium aromaticum*, *Thymus vulgaris* and *Vetiveria zizanioides*. *Origanum vulgare* and *T. vulgaris* had the most noteworthy broad-spectrum minimum inhibitory activity against the ESKAPE pathogens in the studies reviewed, as well as the two most investigated EOs in AMR research (Boskovic et al. 2015, dos Santos

Rodrigues et al. 2017, Leyva-López et al. 2017, Ebani et al. 2023). *Origanum vulgare* had the most data on *P. aeruginosa*, while the most research on *T. vulgaris* focused on *S. aureus*.

*Pelargonium graveolens* was considered to have strong minimum inhibitory activity against *K. pneumoniae* and *P. aeruginosa*, with a MIC value of 12.80 mg/mL (Prabuseenivasan et al. 2006). However, recent studies support the classification of minimum inhibitory activities of EOs as mentioned by Orchard et al. (2017), which identifies noteworthy inhibition at MIC values of  $\leq 1.00$  mg/mL (Chaftar et al. 2016, Amin et al. 2023). Despite these findings, discrepancies in the classification of EOs MIC values persist between journals. These inconsistencies underscore the need to take into account various research results and approaches when assessing the effectiveness of EOs in inhibiting growth. It also emphasizes the need to standardize testing procedures and ensure uniformity in reporting MIC values across studies for more reliable comparisons and conclusions.

Essential oils have demonstrated their ability to inhibit the growth of MDR pathogenic bacteria, indicating their potential as viable alternatives to conventional antibacterial drugs (Nazzaro et al. 2013). Iseppi et al. (2020) reported that *M. alternifolia* (0.02 mg/mL) and *T. vulgaris* (0.03 mg/mL) exhibited notable minimum inhibitory activity against extended-spectrum  $\beta$ -lactamase (ESBL)-producing *K. pneumoniae*, which is associated with severe infections. Fimbres-García et al. (2022) mentioned specific genes that mutate leading to the increased resistance found in *A. baumannii*. It was found that *O. vulgare* was able to withstand these resistant genes and showed superior results when combined with colistin (16x lower than the MIC) (Fimbres-García et al. 2022). This evidence demonstrates the extensive scope of EOs in addressing multi-drug resistance and showcases their potential for synergistic effects. Moreover, EOs demonstrated effectiveness in combatting specific genetic mutations, thus implying their ability to target mechanisms that are typically resistant to conventional antibiotics. These findings suggest that future research should prioritize *in vivo* and clinical studies utilizing EOs in AMR.

**Table 2.1.** Minimum inhibitory concentrations of 23 essential oils against the ESKAPE pathogens.

| Essential oils  | Main compounds   | Previous inhibitory activity                     |                   |                               |
|---|--|--|-------------------|-------------------------------|
|   |  | Pathogen   | MIC value         | Reference                     |
| <i>Allium sativum</i> L.<br>(garlic)                  | Not specified  | <i>P. aeruginosa</i> susceptible clinical strain | 0.02 mg/mL        | Tsao and Yin (2001)           |
|   |  | <i>P. aeruginosa</i> resistant clinical strain   | 0.01 - 0.02 mg/mL |                               |
|   |  | <i>K. pneumoniae</i> resistant clinical strain   | 0.03 mg/mL        |                               |
|   | Diallyl sulfide (57.10%)<br>Diallyl trisulfide (19.50%)  | <i>P. aeruginosa</i> (ATCC 27853)                | 0.13 mg/mL        | Orchard et al. (2017)         |
| <i>Carum carvi</i> L.<br>(caraway)                    | Limonene (34.00%)<br>Carvone (64.10%)  | Resistant <i>K. pneumoniae</i>                   | 1.00 mg/mL        | Singh (2022)                  |
| <i>Cinnamomum verum</i><br>J.Presl<br>(cinnamon leaf) | Cinnamaldehyde (61.50%)<br>β-Caryophyllene (6.80%)<br>Cinnamyl acetate (6.50%)<br>Eugenol (3.70%)<br>β-Phellandene (3.70%) | GMRSA (ATCC 33592)                               | 0.13 mg/mL        | Orchard et al. (2017)         |
|   |  | <i>P. aeruginosa</i> (ATCC 27853)                | 0.50 mg/mL        |                               |
|   |  | <i>S. aureus</i> (ATCC 25923)                    | 0.25 mg/mL        | Orchard et al. (2018)         |
|   |  | MRSA (ATCC 43300)                                | 0.50 mg/mL        |                               |
| <i>Cinnamomum zeylanicum</i> Blume<br>(cinnamon bark) | Eugenol (78.40%)   | <i>P. aeruginosa</i> (ATCC 27853)                | 1.00 mg/mL        | Orchard et al. (2017)         |
|   |  | <i>S. aureus</i> (ATCC 25923)                    | 1.00 mg/mL        |                               |
|   |  | MRSA (ATCC 43300)                                | 1.00 mg/mL        |                               |
|   |  | GMRSA (ATCC 33592)                               | 1.00 mg/mL        |                               |
|   | Eugenol (84.70%)<br>β-Caryophyllene (6.80%)  | Resistant <i>K. pneumoniae</i>                   | 0.50 mg/mL        | Singh (2022)                  |
|   | Cinnamaldehyde (52.40%)<br>Benzaldehyde (12.31%)<br>Benzoic acid (8.20%)<br>Benzyl alcohol (2.23%)                         | <i>K. pneumoniae</i> (ATCC 15380)                | 3.20 mg/mL        | Prabuseenivasan et al. (2006) |
|   |  | <i>P. aeruginosa</i> (ATCC 27853)                | > 0.80 mg/mL      |                               |
|   |  | <i>S. aureus</i> (ATCC 25923)                    | 3.20 mg/mL        |                               |
|   | *( <i>E</i> )cinnamaldehyde<br>Cinnamoyl<br>( <i>E</i> )-acetate<br>Eugenol  | <i>P. aeruginosa</i> (ATCC 27853)                | 0.50 mg/mL        | Firmino et al. (2018)         |
|   |  | <i>S. aureus</i> (ATCC 6538)                     | 0.50 mg/mL        |                               |
| <i>Commiphora myrrha</i><br>Engl. (myrrh)             | Furanoesudema-1,3-diene (52.90%)<br>Lindestrene (15.80%)   | <i>P. aeruginosa</i> (ATCC 27853)                | 1.00 mg/mL        | Orchard et al. (2017)         |
|   |  | <i>S. aureus</i> (ATCC 25923)                    | 1.00 mg/mL        |                               |
|   |  | MRSA (ATCC 43300)                                | 0.50 mg/mL        |                               |
|   |  | GMRSA (ATCC 33592)                               | 0.25 mg/mL        |                               |
| <i>Cymbopogon citratus</i><br>Stapf (lemongrass)      | Limonene (11.70%)<br>Neral (28.90%)<br>Geranial (42.70%)   | <i>P. aeruginosa</i> (ATCC 27853)                | 1.00 mg/mL        | Orchard et al. (2017)         |
|   |  | <i>S. aureus</i> (ATCC 25923)                    | 1.00 mg/mL        |                               |
|   |  | MRSA (ATCC 43300)                                | 1.00 mg/mL        |                               |

| Essential oils                                      | Main compounds   | Previous inhibitory activity  |                                   |                                       |
|---|--|---|-----------------------------------|---------------------------------------|
|   |  | Pathogen  | MIC value                         | Reference                             |
|   |  | GMRSA (ATCC 33592)  | 0.75 mg/mL                        |                                       |
|   | $\beta$ -myrcene (57.52%)<br>Carveol (13.80%)<br>Citral (12.34%)<br>6-methyl-5-hepten-2-one (8.08%)<br>$\alpha$ -pinene (2.65%)<br>3-penten-2-Ol (2.27%) | <i>K. pneumoniae</i> KKU-CRS1 isolated from patients with chronic rhinosinusitis                                      | 0.10% v/v                         | Khosakueng et al. (2024)              |
|   |  | <i>P. aeruginosa</i> KKU-CRS2 isolated from patients with chronic rhinosinusitis                                      | 3.20% v/v                         |                                       |
| <i>Cymbopogon martinii</i> (palmarosa)              | Geraniol (80.70%)  | <i>P. aeruginosa</i> (ATCC 27853)   | 1.00 mg/mL                        | Orchard et al. (2017)                 |
|   |  | <i>S. aureus</i> (ATCC 25923)   | 0.50 mg/mL                        |                                       |
|   |  | GMRSA (ATCC 33592)  | 1.00 mg/mL                        |                                       |
| <i>Ferula galbaniflua</i> Boiss. & Buhse (galbanum) | $\alpha$ -Pinene (8.30%)<br>$\beta$ -Pinene (54.30%)<br>d-3-Carene (7.80%)   | <i>P. aeruginosa</i> (ATCC 27853)   | 0.66 mg/mL                        | Orchard et al. (2017)                 |
| <i>Foeniculum dulce</i> Mill. (fennel)              | trans-Anethol (80.73%)   | GMRSA (ATCC 33592)  | 1.00 mg/mL                        | Orchard et al. (2018)                 |
|   |  | <i>P. aeruginosa</i> (ATCC 27853)   | 1.00 mg/mL                        |                                       |
|   | trans-Anethol (80.73%)   | <i>K. pneumoniae</i> (ATCC 13883)   | 0.01 mg/mL                        | Leigh-de Rapper and van Vuuren (2020) |
| <i>Laurus nobilis</i> L. (bay)                      | 1,8-cineole (up to 30.80%)<br>Methyl eugenol (15.20 - 15.60%)<br>$\alpha$ -terpinyl acetate (up to 14.50%)   | <i>S. aureus</i> (ATCC 6538)  | 31.25 mg/mL                       | Merghni et al. (2016)                 |
|   |  | Eugenol (54.40%)<br>Myrcene (18.50%)<br>Chavicol (11.50%)   | <i>P. aeruginosa</i> (ATCC 27853) | 1.00 mg/mL                            |
|   | <i>S. aureus</i> (ATCC 25923)  |   | 1.00 mg/mL                        |                                       |
|   | MRSA (ATCC 43300)  |   | 1.00 mg/mL                        |                                       |
|   |  |   | GMRSA (ATCC 33592)                | 1.00 mg/mL                            |
| <i>Lavandula angustifolia</i> Bubani (lavender)     | Not specified  | <i>P. aeruginosa</i> (ATCC 27853)   | 1.60% v/v                         | Özfenerci et al. (2022)               |
|   |  | <i>K. pneumoniae</i> (ATCC 700603)  | 0.40% v/v                         |                                       |
|   |  | <i>S. aureus</i> (ATCC 29213)   | 0.40% v/v                         |                                       |
|   | Linalool (38.74%)  | <i>S. aureus</i> CIP 483  | 0.36 mg/mL                        | Lahkimi et al. (2020)                 |
| <i>Litsea cubeba</i> Pers. (may chang)              | $\alpha$ -Citral (38.28)<br>$\beta$ -Citral (29.29)<br>Cinene (16.53)  | <i>A. baumannii</i> (with $\beta$ -lactam resistance genes, sulfa resistance genes and tetracycline resistance genes) | 1.08 mg/mL                        | Yang et al. (2021)                    |
|   |  | Geraniol (44.60%)<br>Neral (28.80%)   | <i>S. aureus</i> (ATCC 25923)     | 1.00 mg/mL                            |
|   | MRSA (ATCC 43300)  |   | 0.50 mg/mL                        |                                       |
|   | GMRSA (ATCC 33592)   |   | 0.50 mg/mL                        |                                       |
| <i>Melaleuca alternifolia</i> Cheel (tea tree)      | Terpinen-4-ol (43.29%)<br>$\gamma$ -terpinene (20.16%)<br>$\alpha$ -terpinene (8.89%)  | <i>S. aureus</i> (clinical isolate resistant to methicillin)  | 8.00 $\mu$ g/mL                   | (Iseppi et al. 2023)                  |
|   |  | <i>E. faecium</i> (clinical isolate resistant to vancomycin)  | 64.00 $\mu$ g/mL                  |                                       |
|   | Not specified  | MDR <i>P. aeruginosa</i>  | 0.01 to 0.50% w/v                 | Bučková et al. (2018)                 |
|   | *Terpinen-4-ol<br>p-cymene   | <i>A. baumannii</i>   | 0.12 - 0.25% v/v                  | Sakkas et al. (2016)                  |
|   | 1,8-Cineole (5.20%)<br>$\gamma$ -Terpinene (19.50%)  | <i>S. aureus</i> (ATCC 25923)   | 0.05 mg/mL                        | Noumi et al. (2018)                   |

| Essential oils                                      | Main compounds   | Previous inhibitory activity  |                             |                               |
|---|--|---|-----------------------------|-------------------------------|
|   |  | Pathogen  | MIC value                   | Reference                     |
|   | Terpinen-4-ol (40.40%)   | MRSA (ATCC 43300)   | 0.05 mg/mL                  |                               |
|   | Not specified  | Metallo-beta-lactamase (MBL)-producing <i>P. aeruginosa</i>         | 16.00 µg/mL                 | Iseppi et al. (2020)          |
|   | Not specified  | Extended-spectrum β-lactamase (ESBL)-producing <i>K. pneumoniae</i> | 16.00 µg/mL                 |                               |
|   | *Terpinen-4-ol<br>α-terpinene<br>p-cymene<br>(tested 10 different oils from different suppliers) | GMRSA (ATCC 33592)  | 0.50 - 2.50 mg/mL           | Brun et al. (2019)            |
|   |  | <i>P. aeruginosa</i> (ATCC BAA-2108)                                | 0.25 - 2.00 mg/mL           |                               |
|   | Not specified  | <i>A. baumannii</i> (ATCC 19606)                                    | 0.80% v/v                   | Özfenerci et al. (2022)       |
|   |  | <i>K. pneumoniae</i> (ATCC 700603)                                  | 0.80% v/v                   |                               |
|   |  | <i>S. aureus</i> (ATCC 29213)                                       | 0.40% v/v                   |                               |
| <i>Matricaria recutita</i> L. (german chamomile)    | Terpinen-4-ol (44.60%)<br>γ-Terpinene (16.60%)<br>p-Cymene (9.60%)                               | <i>S. aureus</i> (ATCC 25923)                                       | 0.50 mg/mL                  | Orchard et al. (2018)         |
|   |  | GMRSA (ATCC 33592)  | 0.25 mg/mL                  |                               |
|   |  | <i>P. aeruginosa</i> (ATCC 27853)                                   | 1.00 mg/mL                  |                               |
| <i>Ocinum tenuiflorum</i> L. (holy basil)           | Not specified  | <i>K. pneumoniae</i> KCU-CRS1                                       | 0.20 - 0.80% v/v            | Khosakueng et al. (2024)      |
|   |  | <i>P. aeruginosa</i> KCU-CRS2                                       | > 3.20% v/v                 |                               |
|   | Linalool (55.20%)<br>Eugenol (10.40%)  | <i>S. aureus</i> (ATCC 25923)                                       | 0.50 mg/mL                  | Orchard et al. (2018)         |
|   |  | MRSA (ATCC 43300)   | 1.00 mg/mL                  |                               |
|   |  | GMRSA (ATCC 33592)  | 0.25 mg/mL                  |                               |
| <i>P. aeruginosa</i> (ATCC 27853)                   | 1.00 mg/mL   |   |                             |                               |
| <i>Origanum vulgare</i> L. (oregano)                | Thymol (78.21%)  | MDR <i>Enterobacter</i>   | 0.31 ± 0.0 to 5 ± 0.0 µL/mL | Benameur et al. (2022)        |
|   | Not specified  | MDR <i>P. aeruginosa</i>  | 0.01 to 0.50% w/v           | Bučková et al. (2018)         |
|   |  | <i>P. aeruginosa</i>  | 0.03% w/v                   |                               |
|   |  | <i>E. cloacae</i>   | 0.01% w/v                   |                               |
|   | Guaiacol-p-vinil (68.67%)<br>p-Cymene (4.60%)<br>β-ionol (3.16%)                                 | <i>P. aeruginosa</i> PA01   | 0.05 mg/mL                  | Merghni et al. (2022)         |
| <i>S. aureus</i> (ATCC 6538)                        |  | 0.05 mg/mL  |                             |                               |
| *Carvacrol<br>Thymol                                | <i>A. baumannii</i>  | 0.25 - 0.37% v/v  | Sakkas et al. (2016)        |                               |
| <i>Pelargonium graveolens</i> L'Hér (rose geranium) | Not specified  | <i>A. baumannii</i> clinical isolates (XDR-A)                       | 5.00 - 20.00 µL/mL          | Kafa et al. (2022)            |
|   | Not specified  | <i>K. pneumoniae</i> (ATCC 15380)                                   | 12.80 mg/mL                 | Prabuseenivasan et al. (2006) |
|   |  | <i>P. aeruginosa</i> (ATCC 27853)                                   | > 12.80 mg/mL               |                               |
|   |  | <i>S. aureus</i> (ATCC 25923)                                       | > 12.80 mg/mL               |                               |
|   | Citronellol (26.70%)<br>Eudesmol (12.50%)<br>Citronellyl formate (11.50%)<br>Menthone (7.80%)    | <i>S. aureus</i> (ATCC 25923)                                       | 1.00 mg/mL                  | Orchard et al. (2018)         |
| GMRSA (ATCC 33592)                                  |  | 0.25 mg/mL  |                             |                               |

| Essential oils   | Main compounds   | Previous inhibitory activity         |                        |                          |
|--|--|--------------------------------------|------------------------|--------------------------|
|  |  | Pathogen                             | MIC value              | Reference                |
|  | Linalool (7.20%)   | <i>P. aeruginosa</i> (ATCC 27853)    | 1.00 mg/mL             |                          |
| <i>Pogostemon patchouli</i><br>Benth. (patchouli)                      | $\beta$ -Patchoulene (38.30%)<br>$\alpha$ -Bulnesene (13.00%)<br>$\alpha$ -Guaiene (11.90%)                | <i>P. aeruginosa</i> (ATCC 27853)    | 1.00 mg/mL             | Orchard et al. (2017)    |
|  |  | <i>S. aureus</i> (ATCC 25923)        | 0.50 mg/mL             |                          |
|  |  | MRSA (ATCC 43300)                    | 1.00 mg/mL             |                          |
|  |  | GMRSA (ATCC 33592)                   | 0.25 mg/mL             |                          |
|  |  | GMRSA (ATCC 33592)                   | 0.25 mg/mL             | Orchard et al. (2018)    |
|  |  | <i>P. aeruginosa</i> (ATCC 27853)    | 1.00 mg/mL             |                          |
| <i>Rosa damascena</i> Mill.<br>(rose otto)                             | Not specified  | <i>P. aeruginosa</i> PA01            | 0.013 - 0.10% v/v      | Bali et al. (2021)       |
|  |  | <i>S. aureus</i> (ATCC 25923)        | 1.00 mg/mL             | Orchard et al. (2018)    |
|  |  | MRSA (ATCC 43300)                    | 1.00 mg/mL             |                          |
|  |  | GMRSA (ATCC 33592)                   | 0.50 mg/mL             |                          |
|  |  | <i>P. aeruginosa</i> (ATCC 27853)    | 1.00 mg/mL             |                          |
| <i>Santalum austrocaledonicum</i><br>Merr. & L.M.Perry<br>(sandalwood) | $\alpha$ -Santalol (32.10%)<br>cis- $\alpha$ -Santalol (11.30%)<br>4,5,9,10-Dehydroisolongifolene (12.30%) | <i>S. aureus</i> (ATCC 25923)        | 0.50 mg/mL             | Orchard et al. (2017)    |
|  |  | MRSA (ATCC 43300)                    | 0.50 mg/mL             |                          |
|  |  | GMRSA (ATCC 33592)                   | 0.25 mg/mL             |                          |
| <i>Syzygium aromaticum</i> L.<br>(clove)                               | Not specified  | Clinical strains of <i>S. aureus</i> | 0.80 - 1.60 $\mu$ g/mL | Jafri et al. (2014)      |
|  |  | <i>E. cloacae</i>                    | 0.05% w/v              | Bučková et al. (2018)    |
|  |  | <i>P. aeruginosa</i> (ATCC 27853)    | 1.00 mg/mL             | Orchard et al. (2017)    |
|  |  | <i>S. aureus</i> (ATCC 25923)        | 0.88 mg/mL             |                          |
|  |  | MRSA (ATCC 43300)                    | 1.00 mg/mL             |                          |
| GMRSA (ATCC 33592)   | 1.00 mg/mL   |                                      |                        |                          |
| <i>Thymus vulgaris</i> Willk.<br>(thyme)                               | Thymol (18.90%)<br>c-Terpinene (7.20%)<br>p-Cymene (41.00%)  | <i>P. aeruginosa</i> (ATCC 27853)    | 1.00 mg/mL             | Orchard et al. (2017)    |
|  |  | <i>S. aureus</i> (ATCC 25923)        | 1.00 mg/mL             |                          |
|  | *Thymol<br>p-cymene<br>Linalool  | <i>A. baumannii</i>                  | 0.25 - 0.5% v/v        | Sakkas et al. (2016)     |
|  | Thymol (37.00-55.00%)<br>Carvacrol (0.50-5.50%)<br>Linalool (1.50-6.50%)<br>Terpinen-4-ol (0.10-2.50%)     | <i>A. baumannii</i>                  | 0.50 mg/mL             | Frydrysiak et al. (2021) |
|  | Thymol (18.90%)<br>c-Terpinene (7.20%)<br>p-Cymene (41.00%)  | GMRSA (ATCC 33592)                   | 1.00 mg/mL             | Orchard et al. (2017)    |
|  | Not specified  | <i>E. cloacae</i>                    | 0.03% w/v              | Bučková et al. (2018)    |
|  | Thymol (18.90%)<br>$\gamma$ -Terpinene (7.20%)<br>p-Cymene (41.00%)  | Resistant <i>K. pneumoniae</i>       | 0.50 mg/mL             | Singh (2022)             |
|  | Not specified  | <i>A. baumannii</i> (ATCC 19606)     | 0.04% v/v              | Özfeneci et al., 2022    |
|  |  | <i>K. pneumoniae</i> (ATCC 700603)   | 0.08% v/v              |                          |
| <i>S. aureus</i> (ATCC 29213)  |  | 0.04% v/v                            |                        |                          |

| Essential oils                               | Main compounds                            | Previous inhibitory activity  |             |                       |
|--|---|---|-------------|-----------------------|
|  |   | Pathogen  | MIC value   | Reference             |
|  | Not specified                             | MBL-producing <i>P. aeruginosa</i>                                  | 4.00 µg/mL  | Iseppi et al. (2020)  |
|  | Not specified                             | Extended-spectrum β-lactamase (ESBL)-producing <i>K. pneumoniae</i> | 32.00 µg/mL |                       |
| <i>Vetiveria zizanioides</i> Stapf (vetiver) | β -Vetirenene (8.80%)<br>Zizanol (12.80%) | <i>S. aureus</i> (ATCC 25923)                                       | 0.50 mg/mL  | Orchard et al. (2018) |
|  |   | MRSA (ATCC 43300)   | 0.50 mg/mL  |                       |
|  |   | GMRSA (ATCC 33592)  | 0.13 mg/mL  |                       |
|  |   | <i>P. aeruginosa</i> (ATCC 27853)                                   | 1.00 mg/mL  |                       |

\* Indicates where percentages of the specific compounds were not provided.

There are several factors that influence the overall MIC value of an EO, namely chemical composition, source of plant material, method of distillation, and growth environments of the plant prior to distillation (Burt 2004). A study investigating the MIC activity of *C. verum* EO from three different suppliers found that the MIC value against *S. aureus* ATCC 43300 varied between < 0.09, 0.19 and 6.25 µl/mL. When comparing the inhibitory activity of *P. graveolens* in two different studies against the same strain of *P. aeruginosa* ATCC 27853, it was found that the MIC value differed by approximately 11.80 mg/mL (Prabuseenivasan et al. 2006, Orchard et al. 2017). The reason for this variation could not definitively be linked to differences in chemical composition, as the earlier study did not detail this information.

A main contributing factor to the minimum inhibitory activity of EOs comes down to their chemical composition. This was also considered when differences in the MIC values of EOs against the same pathogens were observed. Two highly effective antimicrobial compounds are carvacrol and thymol. Therefore, EOs containing significant percentages of these compounds exhibit notable minimum inhibitory activity (Moshrefi Zenoozi et al., 2022). *Origanum vulgare* is an example of an oil abundant in carvacrol, while *T. vulgaris* is rich in thymol. However, what also needs to be considered is that the minor compounds may be a critical part of the EOs' activity, by acting synergistically with the major compounds (Burt, 2004). There is evidence to support this claim and was found to be true in the case of certain species of *Thymus* and *Origanum* (Paster et al., 1995). This study focused on neat EOs instead of compounds. While numerous studies have reported the minimum inhibitory activity of EOs (Table 2.1), it was necessary to confirm MIC values due to possible chemotypic variation.

The MICs of conventional antibiotics used to treat infections caused by ESKAPE pathogens are often nearly 1000 times lower than the MICs of EOs, regardless of whether the strain being tested is resistant or susceptible to the conventional antibiotic (Van et al. 2022). However, studies have reported instances where EOs exhibit lower MICs than conventional antibiotics (Ghavam et al. 2022). Furthermore, it is generally not recommended to directly compare MICs between different types of antimicrobials due to several reasons, such as differences in their mechanisms of action (single-targeted versus multi-targeted effects), the complexity of EO compositions, and factors related to clinical relevance (pharmacokinetic parameters, bioavailability, etc.), as well as variations in testing method (Yap et al. 2014). Thus, the concentrations that are considered to be noteworthy in EOs are different to what is considered noteworthy in conventional antibiotics.

There are several reviews summarizing the minimum inhibitory activity of EOs, their constituent compounds, as well as various combinations of compounds or EOs (Burt 2004, Lang and Buchbauer 2012, Yap et al. 2014, Orchard et al. 2017, Khameneh et al. 2019, Tariq et al. 2019). A recent review by Panda et al. (2022), aligned with the focus of this study, which centres on exploring the efficacy of EOs against ESKAPE pathogens. Research on EOs and their effectiveness against foodborne pathogens is more prevalent compared to studies on the use of EOs in addressing resistance mechanisms, especially induction of resistance, in the medical field (Alvarez et al. 2012, Camele et al. 2019). This area warrants further investigation as understanding the impact of EOs on bacterial resistance mechanisms is of paramount importance in the advancement of novel antimicrobial strategies within the medical domain (Zsuzsanna et al. 2010).

## **2.2 Essential oil's ability to overcome the emergence of bacterial resistance**

Numerous studies have explored the prospect of using EOs to combat resistance in various micro-organisms. Reviews by Lang and Buchbauer (2012), Valdivieso-Ugarte et al. (2019), and Ma et al. (2020), highlight the broad-spectrum antimicrobial properties of EOs which would possibly address the issue of resistance. Chao et al. (2000) further supports this, demonstrating the inhibitory effects of EOs on a variety of micro-organisms (fungi, bacteria, and viruses). Yap (2014) takes this a step further, discussing the potential of EOs to modify resistance in combination with conventional antibiotics. These studies collectively suggest the

vast potential of EOs as an alternative in the crisis of AMR. Research has highlighted the potential of EOs to enhance the efficacy of antibiotics and inhibit the growth of drug-resistant bacteria (Aelenei et al. 2016, Tariq et al. 2019). Aelenei et al. (2016), specifically discusses the role of EOs in reversing drug resistance in Gram-negative bacteria, which could resolve many issues currently faced in the fight against AMR.

Bacteria, in their constant drive to withstand the effects of antimicrobial agents, actively incorporate multiple mechanisms of resistance. Among these, biofilm formation and QS activity, both of which have garnered significant attention in AMR research (Reichling 2020). Additionally, a highly fundamental characteristic lies in the induction of resistance following exposure to EOs. The consequences resulting from the excessive and improper utilization of conventional antibiotics have given rise to bacterial adaptation, enabling them to withstand the inhibitory impact of these therapies (Stenger et al. 2021). When bacteria are repeatedly exposed to an antimicrobial the outcome could result in adaptation and emergence of resistance against such an antimicrobial agent (Melo et al. 2015). Thus, this study necessitates investigating the effects of exposure to sub-inhibitory concentrations of EOs to identify whether resistance occurs. Sionov and Steinberg (2022) advocate for research to focus on mechanisms such as QS and biofilms as a means of finding a solution to the AMR crisis.

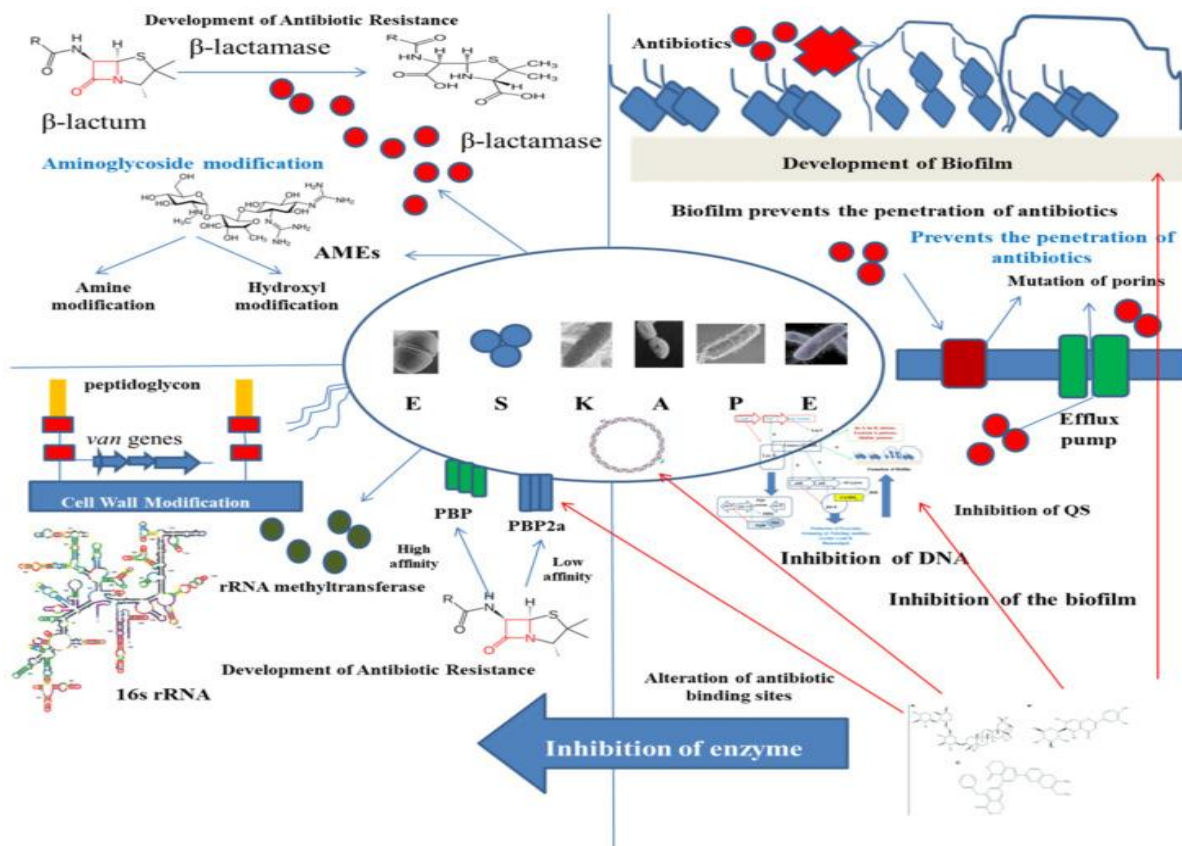
### ***2.2.1 Anti-quorum sensing***

#### *2.2.1.1 The role of quorum sensing on pathogenicity*

Quorum sensing regulates the expression of genes involved in antibiotic resistance mechanisms, such as biofilm formation, allowing bacteria to adapt and survive in the presence of antimicrobial agents (Li and Tian 2012). This phenomenon is extensively responsible for the resistance observed in the ESKAPE pathogens, as well as facilitating inter-species dissemination of resistance among bacterial strains. It is established that the existence of AMR results from the survival of bacteria in the presence of antimicrobial agents, which subsequently undergo evolutionary adaptations to withstand the same mechanisms employed by these agents. Subsequently, the acquired knowledge is shared with other bacterial species, enabling them to confront and withstand the antimicrobial agents, thereby augmenting resistance even further (Sionov and Steinberg 2022). Ultimately, the goal is to identify EOs that possess not only noteworthy broad-spectrum antimicrobial activity but also can hinder QS signalling. The

various mechanisms to encumber QS signalling are demonstrated in Figure 2.1 (Ghosh et al. 2022).

Essential oils present a diverse strategy for reducing bacterial pathogenicity by interfering with QS, an important mechanism for bacterial communication and co-ordination (Ghosh et al. 2022). Essential oils hinder the activation of genes responsible for producing toxins, adhesion molecules, and biofilm formation proteins through various methods such as disrupting autoinducer production, inhibiting QS receptors, and lowering the expression of virulence genes (Kaufmann et al. 2008, Jaramillo-Colorado et al. 2012). This interference also affects the control of biofilm formation, which is crucial for bacterial persistence and virulence. Essential oils either disrupt QS signals or directly interfere with biofilm formation (Adeyemo et al. 2022). As a result, this increases bacteria's vulnerability to these potential antimicrobial agents and host immune defences. Moreover, EOs impact bacterial behaviour by influencing motility, growth, and other physiological processes that impair their ability to cause infections and contribute to pathogenicity (Ahmad et al. 2015).



**Figure 2.1.** Quorum sensing pathways leading to resistance development in the ESKAPE pathogens (Ghosh et al. 2022). Permission to use image granted by the corresponding author.

### 2.2.1.2 Anti-quorum sensing activity of essential oils

Several studies have reported on the anti-QS activities of the 23 EOs, which are the primary focus of this investigation (Table 2.2) (Bai and Vittal 2014, Cáceres et al. 2020, Čobanović et al. 2023). *Carum carvi*, *T. vulgaris* and *O. vulgare* were previously classified as EOs with anti-QS properties, and this was owing to their chemical composition (Raal et al. 2012, Elshafie et al. 2015, Al-Haidari et al. 2016). It was concluded by Alibi et al. (2020), that *C. verum*, *O. majorana*, *S. aromaticum* and *T. vulgaris* had substantial antimicrobial, anti-QS, and biofilm inhibition activities against Gram-negative MDR bacteria. A review reported several EOs, namely *C. zeylanicum*, *C. verum*, *O. vulgare*, *T. vulgaris*, *M. alternifolia*, and *S. aromaticum*, with anti-QS properties (Reichling 2020). A study investigating the anti-QS of *A. sativum*, fed mice garlic which resulted in decreased QS signalling of *P. aeruginosa* (Harjai et al. 2010). Some EOs were tested at high concentrations with very low anti-QS activity (violacein inhibition) (Table 2.2). This was observed for *C. carvi* and *M. alternifolia*, which were both tested at 10.00 mg/mL with < 70.00% violacein inhibition (Noumi et al. 2018, Ghannay et al. 2022). Ten EOs had no data on anti-QS activity regarding violacein inhibition (Table 2.2).

In a comprehensive review, Wu et al. (2024) delved into the necessity of inhibiting QS in order to prevent the formation and regulation mechanisms of biofilms for *S. aureus*. The authors advocated for the use of EO as a prospective antimicrobial tool, emphasizing its potential in combating AMR, by targeting QS. The review also discussed how the utilization of EOs can overcome resistance on account of their multi-component structure and ability to inhibit efflux pumps. The inhibition of efflux pumps prevents antibiotics from being expelled, thereby “reversing” resistance and reintroducing susceptibility of previously resistant antibiotics (Álvarez-Martínez et al. 2021). Overall EOs targeting QS pathways offer the potential to develop innovative antimicrobial strategies against bacterial infections (Ghosh et al. 2022).

There is abundant literature linking QS and biofilm mechanisms, however, many studies only focus on one of the methods (Harjai et al. 2010, Jaramillo-Colorado et al. 2012, Olivero-Verbel et al. 2014, Ahmad et al. 2015, Al-Haidari et al. 2016, Poli et al. 2018, Wang et al. 2019). Fewer studies, however, did incorporate both methods (Li and Tian 2012, Alibi et al.

2020, Adeyemo et al. 2022, Merghni et al. 2022, D'Aquila et al. 2023). Numerous methodologies exist for exploring anti-QS activities, such as the assessment of violacein production, pyocyanin production, elastase activity, swarming motility and specific genes that control QS activity. However, this review specifically concentrated on anti-QS investigations centred on violacein inhibition, due to its relevance to the current study. Investigation into violacein inhibition is a widely utilized method for determining the anti-QS activity of EOs and is closely linked to biofilm formation (Mahumane 2016, Batista et al. 2017, Sionov and Steinberg 2022).

## **2.2.2 Biofilm inhibition**

### *2.2.2.1 The role of biofilm inhibition on pathogenicity*

Micro-organisms within a biofilm have the potential to develop resistance to antibiotics that is significantly greater by a factor of up to 1000, in comparison to planktonic bacteria that exist in a free-living state (Oppenheimer-Shaanan et al. 2013). Furthermore, these micro-organisms possess a remarkable ability to effectively avoid detection and neutralization by the immune system. The emergence of the MRSA strain is largely attributed to the formation of biofilms, which inherently renders it resistant to nearly all  $\beta$ -lactam antibiotics, the largest and most frequently utilized class of antibiotics (Craft et al. 2019). In this context, investigating the potential of EOs as biofilm inhibitors holds great significance in the evaluation of their potential contribution to the development of resistance (Cáceres et al. 2020).

Essential oils are antimicrobials with the ability to change the biofilm matrix which inhibits and prevents its formation (Tariq et al. 2019). Nazzaro et al. (2013) brought up an interesting argument that EOs have a greater effect on preformed biofilms because of how easily they can penetrate them due to the nature of their non-polar components. It was highlighted several times in the previously mentioned review (Nazzaro et al. 2013) that *O. vulgare* has not only proven to be a potent anti-QS agent, but it was also able to prevent and eradicate biofilms. The same argument is made as previously mentioned by Sionov and Steinberg (2022), that hindering QS, which controls biofilm formation, will affect virulence factors leading to resistance.

### 2.2.2.2 *Biofilm inhibitory potential of essential oils*

In recent years there have been several reviews that have conducted thorough analyses of natural products, specifically EOs, that possess notable biofilm inhibitory properties which are highlighted in Table 2.3 (Ćirić et al. 2019, Mulani et al. 2019, Tariq et al. 2019, Cáceres et al. 2020, Yu et al. 2020, El-Tarabily et al. 2021). A review by Reichling (2020), also assessed biofilm inhibition of many EOs, and found that *C. zeylanicum*, *C. verum*, *O. vulgare*, *T. vulgaris*, *M. alternifolia*, *S. aromaticum*, *C. martini*, *C. citratus*, and *L. angustifolia* were all able to inhibit biofilm formation. dos Santos Rodrigues et al. (2017), found that the MIC value of *O. vulgare* EO against a strong biofilm-forming *S. aureus* strain was lower against sessile bacteria than planktonic bacteria.

The potential biofilm inhibitory activity of EOs is thought to be associated with their unique bio-active constituents (Tapia-Rodriguez et al. 2023). The synergism between compounds, including those present in smaller quantities, exhibits an effect that results in an overall robust antimicrobial activity (Kim et al. 2016). This trend also holds true for the inhibition of biofilm formation, as the synergistic behaviour yields a more effective inhibition of biofilm compared to the individual compounds when tested against pathogenic bacteria (Burt 2004, Masyita et al. 2022). In some cases, the individual compounds may not exhibit optimal performance and may cause damage to the microbial cell membrane, which proves to be less effective in terms of biofilm inhibition (Ghosh et al. 2022). Both EOs and EO compounds offer valuable avenues for research, typically dictated by research objectives, availability of resources, target micro-organisms, and the desired specificity or potency of biofilm inhibitory effects. The limitations of several antibiotics in preventing or eradicating biofilms are attributed to their single-cell targeted action, as highlighted by Ali Mohammed et al. 2013.

## 2.2.3 *Induction of resistance*

### 2.2.3.1 *Mechanisms bacteria use to induce resistance*

Bacteria have the ability to develop resistance to antimicrobial agents through a range of methods, such as genetic changes, horizontal gene transfer, and adaptive reactions to environmental pressures (Munita and Arias 2016). In addition to these, bacteria also utilize QS and biofilm formation as tactics to bolster their resistance capabilities (Händel et al. 2014).

**Table 2.2.** Anti-quorum sensing studies on the 23 essential oils investigated in this study.

| Essential oil                                | Biosensor strain               | Results                           | Concentration | Method   | Reference                |
|--|--------------------------------|-----------------------------------|---------------|--|--------------------------|
| <i>Allium sativum</i> (garlic)               | No studies found*              |                                   |               |  |                          |
| <i>Carum carvi</i> (caraway)                 | <i>C. violaceum</i>            | 47.57%                            | 10.00 mg/mL   | Microtiter plate                                       | Ghannay et al. (2022)    |
| <i>Cinnamomum verum</i> (cinnamon leaf)      | <i>C. violaceum</i>            | 91.68%                            | 1.00 mg/mL    | Macrodilution  | Alibi et al. (2020)      |
|  | <i>C. violaceum</i>            | 0.01% there was QS inhibition     | 0.01%         | Microdilution  | Kim et al. (2015)        |
| <i>Cinnamomum zeylanicum</i> (cinnamon bark) | No studies found*              |                                   |               |  |                          |
| <i>Commiphora myrrha</i> (myrrh)             | No studies found*              |                                   |               |  |                          |
| <i>Cymbopogon citratus</i> (lemongrass)      | <i>C. violaceum</i>            | 91.40%                            | 0.0013% v/v   | Microdilution method, quantifying violacein production | Khosakueng et al. (2024) |
| <i>Cymbopogon martinii</i> (palmarosa)       | No studies found*              |                                   |               |  |                          |
| <i>Ferula galbaniflua</i> (galbanum)         | No studies found*              |                                   |               |  |                          |
| <i>Foeniculum dulce</i> (fennel)             | No studies found*              |                                   |               |  |                          |
| <i>Laurus nobilis</i> (bay)                  | <i>C. violaceum</i>            | No inhibition (qualitative)       | 0.01% v/v     | Disc diffusion, screening assay                        | Eris and Ulusoy (2013)   |
| <i>Lavandula angustifolia</i> (lavender)     | <i>C. violaceum</i>            | Potent QS inhibitor               | Not specified | Not specified: quantified violacein production         | Szabó et al. (2010)      |
|  | <i>C. violaceum</i> ATCC 12472 | 99.20%                            | 0.04% v/v     | Violacein production                                   | Özfenerci et al. (2022)  |
|  | <i>C. violaceum</i>            | Very low inhibition (qualitative) | 0.01% v/v     | Disc diffusion, screening assay                        | Eris and Ulusoy (2013)   |
| <i>Litsea cubeba</i> (may chang)             | No studies found*              |                                   |               |  |                          |
| <i>Melaleuca alternifolia</i> (tea tree)     | <i>C. violaceum</i> ATCC 12472 | 69.30%                            | 10.00 mg/mL   | Microtiter plate                                       | Noumi et al. (2018)      |
|  | <i>C. violaceum</i> ATCC 12472 | 99.38%                            | 0.04% v/v     | Violacein production                                   | Özfenerci et al. (2022)  |

| Essential oil                                  | Biosensor strain                        | Results             | Concentration      | Method   | Reference                   |
|--|---|---------------------|--------------------|--|-----------------------------|
|  | <i>C. violaceum</i> ATCC 12472          | 100.00%             | 2.00 mg/mL         | Microtiter plate                               | Alfred Ngenge et al. (2021) |
| <i>Matricaria recutita</i> (german chamomile)  | <i>C. violaceum</i> (different strains) | 32.00 - 75.00%      | 0.0025% v/v        | Flask incubation assay                         | Eris and Ulusoy (2013)      |
|  | <i>C. violaceum</i>                     | Ineffective         | Not specified      | Not specified: quantified violacein production | Szabó et al. (2010)         |
| <i>Ocimum tenuiflorum</i> (holy basil)         | No studies found*                       |                     |                    |  |                             |
| <i>Origanum vulgare</i> (oregano)              | <i>C. violaceum</i> ATCC 12472          | 72.70%              | 0.048 mg/mL        | Violacein production                           | Merghni et al. (2022)       |
|  | <i>C. violaceum</i> CV026               | 59.31%              | 0.04 mg/mL         | Violacein production                           | Singh and Agarwal (2021)    |
| <i>Pelargonium graveolens</i> (rose geranium)  | <i>C. violaceum</i> ATCC 12472          | Potent QS inhibitor | Not specified      | Not specified: quantified violacein production | Szabó et al. (2010)         |
| <i>Pogostemon patchouli</i> (patchouli)        | <i>C. violaceum</i> 12472               | 94.37%              | 1.25% v/v          | Not specified: quantified violacein production | Mansuri et al. (2022)       |
| <i>Rosa damascena</i> (rose otto)              | <i>C. violaceum</i>                     | 98.04%              | sub-MIC: 0.01% v/v | Not specified: quantified violacein production | Bali et al. (2021)          |
|  | <i>C. violaceum</i> (different strains) | 43.00 - 80.00%      | 0.0025% v/v        | Flask incubation assay                         | Eris and Ulusoy (2013)      |
| <i>Santalum austrocaledonicum</i> (sandalwood) | No studies found*                       |                     |                    |  |                             |
| <i>Syzygium aromaticum</i> (clove)             | <i>C. violaceum</i>                     | 84.13%              | 1.00 mg/mL         | Macrodilution                                  | Alibi et al. (2020)         |
|  | <i>C. violaceum</i>                     | 92.30%              | 0.16% v/v          | Serial dilution                                | Khan et al. (2009)          |
|  | <i>C. violaceum</i> (different strains) | 39.00 - 80.00%      | 0.0025% v/v        | Flask incubation assay                         | Eris and Ulusoy (2013)      |
| <i>Thymus vulgaris</i> (thyme)                 | <i>C. violaceum</i>                     | 99.41%              | 1.00 mg/mL         | Macrodilution                                  | Alibi et al. (2020)         |
|  | <i>C. violaceum</i> ATCC 12472          | 92.28%              | 0.04% v/v          | Violacein production                           | Özfenerci et al. (2022)     |
| <i>Vetiveria zizanioides</i> (vetiver)         | No studies found*                       |                     |                    |  |                             |

\*No studies specifically looking at violacein inhibition, however, studies are evident on extracts, varying methods or *in vivo* investigations.

**Table 2.3.** Known biofilm inhibition studies on essential oils from this study.

| Essential oils                                  | Pathogen                                   | Results                      | Concentration           | Reference                 |
|---|--|------------------------------|-------------------------|---------------------------|
| <i>Allium sativum</i><br>(garlic)               | MDR <i>Salmonella typhimurium</i> isolates | Optical density = 1.67-1.74, | 0.49 µL/mL              | Hakimi Alni et al. (2020) |
|   | <i>A. baumannii</i> ATCC 19606             | 18.59 - 57.34%               | 10 - 20 µL/mL           | Nazzaro et al. (2022)     |
|   | <i>S. aureus</i> ATCC 25923                | 16.70 - 63.18%               | 10 - 20 µL/mL           |                           |
|   | <i>S. aureus</i> ATCC 6538                 | -1.00%                       | 0.01% v/v               | Lee et al. (2014)         |
| <i>Carum carvi L.</i><br>(caraway)              | <i>P. aeruginosa</i> PAO1                  | 60.00 - 72.00%               | 1.00% v/v               | Fekry et al. (2022)       |
|   | MRSA ATCC 43300                            | 100.00%                      | 1.28%                   | Liu et al. (2023)         |
|   | <i>Vibrio</i> spp. Strains                 | < 50.00%                     | 0.02 - 0.18 mg/mL       | Ghannay et al. (2022)     |
|   | <i>S. aureus</i> ATCC 6538                 | -15.00%                      | 0.01% v/v               | Lee et al. (2014)         |
| <i>Cinnamomum verum</i><br>(cinnamon leaf)      | <i>K. pneumoniae</i> clinical isolates     | 95.32 - 98.00%               | MIC/2                   | Alibi et al. (2020)       |
|   | <i>A. baumannii</i> clinical isolates      | 93.85 - 98.67%               | MIC/2                   |                           |
|   | <i>P. aeruginosa</i> clinical isolates     | 97.78 - 99.36%               | MIC/2                   |                           |
|   | <i>S. aureus</i> clinical isolates         | 97.30 - 99.51%               | MIC/2                   |                           |
|   | <i>P. aeruginosa</i> PAO1                  | 96.00%                       | 0.05%                   | Kim et al. (2015)         |
| <i>Cinnamomum zeylanicum</i><br>(cinnamon bark) | <i>E. coli</i> ATCC 8739                   | 65.73 - 72.38%               | 0.05 - 2.00 mg/mL       | Singh (2022)              |
|   | <i>K. pneumoniae</i> ATCC 13883            | 47.98 - 53.59%               | 0.05 - 2.00 mg/mL       |                           |
|   | <i>S. aureus</i>                           | 45.30 - 74.70%               | 0.11 mg/mL              | Budri et al. (2015)       |
|   | <i>Escherichia coli</i> ATCC 25922         | 92.00%                       | 1.92% (v/v)             | Millezi et al. (2019)     |
| <i>Commiphora myrrha</i><br>(myrrh)             | <i>S. aureus</i> ATCC 6538                 | 89.00%                       | 0.01% v/v               | Lee et al. (2014)         |
| <i>Cymbopogon citratus</i><br>(lemongrass)      | <i>S. aureus</i> ATCC 6538                 | 54.00%                       | 0.01% v/v               | Lee et al. (2014)         |
|   | <i>K. pneumoniae</i> KKU-CRS1              | 77.00%                       | MIC/2 (MIC = 0.10% v/v) | Khosakueng et al. (2024)  |
|   | <i>P. aeruginosa</i> KKU-CRS2              | 91.10%                       | MIC/2 (MIC = 3.20% v/v) |                           |
| <i>Cymbopogon 24artini</i><br>(palmarosa)       | <i>S. aureus</i> ATCC 6538                 | 93.00%                       | 0.01% v/v               | Lee et al. (2014)         |
| <i>Ferula galbaniflua</i><br>(galbanum)         | No studies found                           |                              |                         |                           |

| Essential oils                                | Pathogen   | Results                  | Concentration                      | Reference               |
|---|--|--------------------------|------------------------------------|-------------------------|
| <i>Foeniculum dulce</i> (fennel)              | No studies found   |                          |                                    |                         |
| <i>Laurus nobilis</i> (bay)                   | <i>S. aureus</i> ATCC 6538   | 95.38- 96.40%            | 31.25 mg/mL                        | Merghni et al. (2016)   |
|   | <i>S. aureus</i> clinical isolates   | 78.92 - 93.00%           | 31.25 mg/mL                        |                         |
|   | <i>P. aeruginosa</i> ATCC 9027   | 48.00%                   | 125 µg/mL                          | Bektas et al. (2023)    |
| <i>Lavandula angustifolia</i> (lavender)      | <i>P. aeruginosa</i> ATCC 27853  | 21.80%                   | 0.01 - 0.10% v/v                   | Özfenerci et al. (2022) |
|   | <i>P. aeruginosa</i> PAO1  | 65.50%                   | 0.01 - 0.10% v/v                   |                         |
|   | <i>A. baumannii</i> ATCC 19606   | 33.00%                   | 0.01 - 0.10% v/v                   |                         |
|   | <i>S. aureus</i> ATCC 29213  | 61.30%                   | 0.01 - 0.10% v/v                   |                         |
|   | <i>K. pneumoniae</i> ATCC 700603   | 40.00%                   | 0.01 - 0.10% v/v                   |                         |
|   | <i>S. aureus</i> ATCC 6538   | 56.00%                   | 0.01% v/v                          | Lee et al. (2014)       |
|   | <i>S. aureus</i> ATCC 6538   | 26.00%                   | 0.01% v/v                          | Lee et al. (2014)       |
| <i>Litsea cubeba</i> (may chang)              | <i>Vibrio parahaemolyticus</i>   | 64.75%                   | 1024.00 µg/mL                      | Li et al. (2022)        |
|   | <i>A. baumannii</i> (with β-lactam resistance genes, sulfa resistance genes and tetracycline resistance genes) | Great biofilm inhibition | 1/64 to 1/2 MIC (MIC = 1.08 mg/mL) | Yang et al. (2021)      |
|   | <i>H. pylori</i>   | Great biofilm inhibition | (0.50 x MIC) - (0.75 x MIC)        | Hung et al. (2023)      |
|   | <i>S. aureus</i> ATCC 6538   | 55.00%                   | 0.01% v/v                          | Lee et al. (2014)       |
|   | <i>S. aureus</i> (clinical isolate resistant to methicillin)   | 40.82%                   | 8.00 µg/mL                         | Iseppi et al. (2023)    |
| <i>Melaleuca alternifolia</i> (tea tree)      | <i>P. aeruginosa</i> ATCC 27853  | 20.80%                   | 0.01 - 0.10% v/v                   | Özfenerci et al. (2022) |
|   | <i>P. aeruginosa</i> PAO1  | 58.10%                   | 0.01 - 0.10% v/v                   |                         |
|   | <i>A. baumannii</i> ATCC 19606   | 28.02%                   | 0.01 - 0.10% v/v                   |                         |
|   | <i>S. aureus</i> ATCC 29213  | 61.30%                   | 0.10% v/v                          |                         |
|   | <i>K. pneumoniae</i> ATCC 700603   | 20.03%                   | 0.01 - 0.10% v/v                   |                         |
|   | <i>P. aeruginosa</i> PAO1  | 58.00%                   | 0.01% v/v                          |                         |
| <i>Matricaria recutita</i> (german chamomile) | <i>P. aeruginosa</i> PAO1  | 58.00%                   | 0.01% v/v                          | Eris and Ulusoy (2013)  |
| <i>Ocimum tenuiflorum</i> (holy basil)        | <i>C. albicans</i> ATCC 10231  | approximately 40.00%     | 1.25 µl/mL                         | Piras et al. (2018)     |
| <i>Origanum vulgare</i> (oregano)             | <i>S. aureus</i> ATCC 6538   | 77.00%                   | 0.01% v/v                          | Lee et al. (2014)       |
|   | <i>S. aureus</i> ATCC 6538   | > 60.00%                 | 0.006 - 0.018 mg/mL*               | Merghni et al. (2022)   |

| Essential oils                                 | Pathogen   | Results                        | Concentration     | Reference                       |
|--|--|--------------------------------|-------------------|---------------------------------|
|  | <i>S. aureus</i> clinical isolates                       | 78.00%                         | 0.10% v/v         | Kryvtsova et al. (2020)         |
|  | MRSA   | 95.71%                         | 0.09 mg/mL        | Ersanli et al. (2023)           |
| <i>Pelargonium graveolens</i> (rose geranium)  | <i>Acinetobacter baumannii</i> clinical isolates (XDR-A) | 48.00 - 90.00%                 | < 20.00 µl/mL     | Kafa et al. (2022)              |
| <i>Pogostemon patchouli</i> (patchouli)        | <i>S. aureus</i> ATCC 6538                               | 75.00%                         | 0.01% v/v         | Lee et al. (2014)               |
|  | <i>P. aeruginosa</i> PA01                                | 88.13%                         | 1.25% v/v         | Mansuri et al. (2022)           |
|  | <i>Xyllela fastidiosa</i>                                | 50.00%                         | 250 and 125 µg/mL | Brentini Santiago et al. (2018) |
| <i>Rosa damascena</i> (rose otto)              | <i>P. aeruginosa</i> PA01                                | 93.07%                         | sub-MIC: 0.2% v/v | Bali et al. (2021)              |
|  | <i>P. aeruginosa</i> PA01                                | 53.00%                         | 0.0025% v/v       | Eris and Ulusoy (2013)          |
| <i>Santalum austrocaledonicum</i> (sandalwood) | <i>S. aureus</i> ATCC 6538                               | <i>Santalum album</i> = 94.00% | 0.01% v/v         | Lee et al. (2014)               |
|  | <i>E. coli</i> ATCC 8739                                 | 39.59 - 45.62%                 | 0.05 - 2.00 mg/mL | Singh (2022)                    |
|  | <i>K. pneumoniae</i> ATCC 13883                          | 29.36 - 45.27%                 | 0.05 - 2.00 mg/mL |                                 |
| <i>Syzygium aromaticum</i> (clove)             | <i>K. pneumoniae</i> clinical isolates                   | 77.13 - 92.63%                 | MIC/2             | Alibi et al. (2020)             |
|  | <i>A. baumannii</i> clinical isolates                    | 88.01 - 91.88%                 | MIC/2             |                                 |
|  | <i>P. aeruginosa</i> clinical isolates                   | 92.71 - 96.97%                 | MIC/2             |                                 |
|  | <i>S. aureus</i> clinical isolates                       | 90.89 - 96.47%                 | MIC/2             |                                 |
|  | <i>E. coli</i> ATCC 8739                                 | 72.27 - 74.81%                 | 0.05 - 2.00 mg/mL | Singh (2022)                    |
|  | <i>K. pneumoniae</i> ATCC 13883                          | 43.86 - 54.57%                 | 0.05 - 2.00 mg/mL |                                 |
|  | <i>P. aeruginosa</i> PA01                                | 53.00%                         | 0.0025% v/v       | Eris and Ulusoy (2013)          |
|  | <i>S. aureus</i> ATCC 6538                               | 74.00%                         | 0.01% v/v         | Lee et al. (2014)               |
| <i>Thymus vulgaris</i> (thyme)                 | <i>K. pneumoniae</i> clinical isolates                   | 80.44 - 93.68%                 | MIC/2             | Alibi et al. (2020)             |
|  | <i>A. baumannii</i> clinical isolates                    | 81.28 - 92.33%                 | MIC/2             |                                 |
|  | <i>P. aeruginosa</i> clinical isolates                   | 91.17 - 92.94%                 | MIC/2             |                                 |
|  | <i>S. aureus</i> clinical isolates                       | 89.66 - 93.59%                 | MIC/2             |                                 |
|  | <i>S. aureus</i> ATCC 6538                               | 78.00%                         | 0.01% v/v         | Lee et al. (2014)               |
|  | <i>E. coli</i> ATCC 8739                                 | 73.63 - 80.47%                 | 0.05 - 2.00 mg/mL | Singh (2022)                    |
|  | <i>K. pneumoniae</i> ATCC 13883                          | 61.12 - 64.71%                 | 0.05 - 2.00 mg/mL |                                 |

| Essential oils                            | Pathogen                         | Results        | Concentration     | Reference                  |
|---|----------------------------------|----------------|-------------------|----------------------------|
|   | <i>P. aeruginosa</i> ATCC 27853  | 67.67%         | 0.01 - 0.10% v/v  | Özfenerci et al.<br>(2022) |
|   | <i>P. aeruginosa</i> PAO1        | 63.40%         | 0.01 - 0.10% v/v  |                            |
|   | <i>A. baumannii</i> ATCC 19606   | 40.00%         | 0.01 - 0.10% v/v  |                            |
|   | <i>S. aureus</i> ATCC 29213      | 58.55%         | 0.01 - 0.10% v/v  |                            |
|   | <i>K. pneumoniae</i> ATCC 700603 | 47.04%         | 0.01 - 0.10% v/v  |                            |
| <i>Vetiveria zizanioides</i><br>(vetiver) | <i>E. coli</i> ATCC 8739         | 57.40 - 65.72% | 0.05 - 2.00 mg/mL | Singh (2022)               |
|   | <i>K. pneumoniae</i> ATCC 13883  | 61.38%         | 2.00 mg/mL        |                            |

The microtiter plate assay was used for all studies except those \* where biofilm attachment assay was used.

Bacterial mutations in DNA can lead to resistance against antimicrobial agents, either through spontaneous occurrence or as a reaction to selective pressure from exposure to these agents (Mutuku et al. 2022). These mutations can affect various cellular components, such as enzymes targeted by antibiotics or membrane transport proteins, resulting in reduced effectiveness of the antibiotic (Speck et al. 2020). Horizontal gene transfer can occur through mechanisms like conjugation, transformation, and transduction, where bacteria are able to acquire resistance genes from other bacterial species. These acquired genes may encode enzymes that deactivate antibiotics, modify antibiotic targets, or enhance efflux mechanisms for expelling antibiotics from the cell (Jiang et al. 2022, Michaelis and Grohmann 2023). Efflux pumps actively expel antimicrobial agents from within the cell, decreasing their effectiveness by reducing their concentration. Increased expression of efflux pump genes can provide resistance against various antimicrobial agents (Agreles et al. 2021).

There has been a noticeable increase in the emergence of resistant pathogens to new antibiotics, surpassing the rate witnessed in previous years (Akram et al. 2023). Given the notable antimicrobial activity demonstrated by EOs, there is a possibility that they could serve as a potential alternative or adjunct to conventional antibiotics. It is crucial to acknowledge the potential role of EOs in the development of resistance. As demonstrated in Figure 2.2, resistance to conventional antibiotics occurred within a few years after it was introduced (Stennett et al. 2022, Helmy et al. 2023). The benefits of the utilization of EOs must outweigh the associated risks, such as an increased rate of resistance development against EOs and their impact on conventional antibiotics when considering them as a viable long-term solution to the prevailing crisis of AMR. While studies on the induction of resistance have been conducted on *M. alternifolia* by Dalio et al. (2020), research is scarce for this subject matter in relation to many other EOs. The aforementioned study conducted research against *Fusarium* wilt in bananas and *Xanthomonas* infection in tomato plants. It is important to understand that exposure to EOs repeatedly puts the bacteria under stress, and continuous stress leads to adaptations (Conte et al., 2000, Garner et al., 2006, Shaw et al., 2008).

#### 2.2.3.2 Essential oils and induction of bacterial resistance

A study in 2012, investigated the inclination of the clinical isolates of *Serratia marcescens*, to acquire resistance against *O. vulgare* and *C. zeylanicum* and various antibiotics subsequent to repeated exposure to these EOs (Becerril et al. 2012). It was found that exposure

to *O. vulgare* induced resistance to nalidixic acid, where the MIC value increased from 2.00 mg/L to 16 mg/L between the original MIC and the 20<sup>th</sup> passage. Furthermore, increases of one to two serial dilutions were seen when the pathogen was exposed to tetracycline, minocycline, ciprofloxacin, and chloramphenicol between the original MIC value and the 20<sup>th</sup> and 50<sup>th</sup> passage.

| Year antibiotic was introduced | Antibiotic      | Year antibiotic resistance was detected |
|--------------------------------|-----------------|---|
| 1935                           | Sulfonamides    | 1942                                    |
| 1941                           | Penicillin      | 1945                                    |
| 1944                           | Streptomycin    | 1947                                    |
| 1945                           | Cephalosporins  | 1967                                    |
| 1946                           | Aminoglycosides | 1946                                    |
| 1948                           | Tetracyclines   | 1953                                    |
| 1952                           | Macrolides      | 1952                                    |
| 1983                           | Carbapenems     | 1986                                    |
| 1984                           | Monobactams     | 1984                                    |
| 1999                           | Linezolid       | 2001                                    |
|                                | All antibiotics | 2017                                    |

**Figure 2.2.** The emergence of resistance after the introduction of the antibiotic from 1935 to 2017 (Revised from Stennett et al. (2022) and Helmy et al. (2023)).

It was also reported by this study that *Proteus mirabilis* had increased susceptibility to ampicillin due to exposure to *O. vulgare* between the original MIC and the 20<sup>th</sup> passage. This study used the disc diffusion method through the 50 passages and conducted the microdilution assay only at the 20<sup>th</sup> and 50<sup>th</sup> passages. This does not give a full account of the changes and induction of resistance of *O. vulgare* on the MIC value of itself and the conventional antibiotics over the 50 passages (Becerril et al. 2012). Nevertheless, this study offers significant insights into the potential development of resistance and increased susceptibility by pathogens, following repeated exposure to EOs. Conducting further research using comprehensive methodologies, such as the microdilution assay and gene sequencing, across all passages would enhance our understanding of how EOs affect microbial resistance profiles.

Another investigation studying the tolerance of *E. coli*, a parent strain, and a multiple antibiotic resistant (MAR) phenotype, to *M. alternifolia*, found that the differences in the MIC were not significant enough to conclude different degrees of resistance between MAR phenotypes and the parent strain. The reason for incorporating a MAR phenotype was to examine how prior exposure to an antibiotic such as tetracycline and chloramphenicol will affect the MIC of *M. alternifolia* (Gustafson 2001).

Hammer et al. (2008) analysed resistance by evaluating the frequency of mutants and concluded that single-step mutations as a result of exposure to *M. alternifolia* during the late-exponential phase of growth are unlikely to occur. In other words, resistance is unlikely to occur. It was suggested that because several compounds from *M. alternifolia* possess antimicrobial properties with slightly different mechanisms of action, bacteria would have to acquire multiple resistance mechanisms simultaneously in order to counter all these processes (Hammer et al. 2008).

It is documented that exposure to stress can lead to alteration of genes in bacteria which can lead to resistance emergence (Bikels-Goshen et al. 2010). A study tested this, by assessing the impact of the *Melissa officinalis* essential oil on *Listeria monocytogenes* and the ability to tolerate stressful environments. A preliminary treatment with the EO was carried out, followed by exposure to challenging conditions such as elevated temperature, low pH, osmotic stress, and dehydration. This was followed by determining if there were incidents of cross-resistance to the antibiotics tested, due to prior exposure to the EO. The pre-exposure of *L. monocytogenes* to sub-inhibitory levels of the EO (0.125 µL/mL) did not induce high tolerance to stressors or cross-resistance in the antibiotics, such as erythromycin, tetracycline, cefotaxime, and ampicillin (Carvalho et al. 2023).

When examining the impact of eugenol (an EO-derived compound) from *S. aromaticum* on the resistance development of three conventional antibiotics, it was found that there were no changes in the antibiotic susceptibility profiles against four strains of *Helicobacter pylori* (Elbestawy et al. 2023). This indicates that the tested bacterial strains did not develop resistance to antibiotics after multiple exposures to eugenol at a sub-inhibitory concentration of 12.00 µg/mL for 24 hrs (Elbestawy et al. 2023). Another study investigated single and multi-step resistance, specifically examining if mutants arise due to exposure to *M. alternifolia* and one of the major compounds, terpinen-4-ol, against *S. aureus* and *E. coli* (Hammer et al. 2012). It

was reported that there were spikes in the MIC values of vancomycin and mupirocin over the 6-day experimental period. Furthermore, it was observed that the MIC value of the EO increased at days 4 and 5, before returning to the original MIC value (Hammer et al. 2012).

When examining the impact of EOs on resistance, it was noted in the literature that there are multiple factors involved in resistance development. These include the frequency of mutants, upregulation of specific genes, and whether a single-step or multi-step approach is involved. Additionally, several variables can significantly influence the outcomes, such as the duration and growth phase during which exposure to EO occurs (Gustafson 2001, Hammer et al. 2008, Becerril et al. 2012, Hammer et al. 2012). It is generally thought that continuous exposure to an antimicrobial agent may elicit resistance as bacteria adapt by mutating, thereby withstanding the effects of the antimicrobial agent (Munita and Arias 2016). In contrast to this belief, a study has discovered that exposure to *Cinnamomum zeylanicum* EO can disrupt bacterial gene expression and RNA synthesis, as well as modulate the activity of efflux pumps. These findings support the potential of *C. zeylanicum* to act as an adjunct treatment alongside current antibiotic therapies (Coşeriu et al. 2023). Furthermore, it has been documented that the modulation of efflux pumps can lead to the reversal of antibiotic resistance (Agreles et al. 2021). Nelson (2000), investigated the potential of *M. alternifolia* in eradicating MRSA, through exposure to the EO. It was reported that after inducing the methicillin-resistant genes in *S. aureus* the MIC of the EO remained the same on day one and day 90 for three of the five strains. However, for two of the *S. aureus* clinical strains, there was a substantial decrease in the MIC value between day one and day 90, suggesting that MRSA may become more susceptible to *M. alternifolia* after repeated exposure. Nelson (2000) suggested that it was inevitable for resistance to *M. alternifolia* to develop. This phenomenon may be apparent in other EOs not previously studied.

The phenomenon of AMR not only jeopardizes individual patient outcomes but also poses a grave risk to public health by potentially turning once-manageable infections into life-threatening ones (Ventola 2015, Manyi-Loh et al. 2018). As mentioned in Chapter 1, the outbreak of COVID-19 has led to a surge in the utilization of antibiotics, consequently exerting selective pressure on bacterial populations (Taleb et al. 2023). This pressure confers a competitive advantage to resistant strains, resulting in the elimination of non-resistant bacteria. Over time, the surviving resistant bacteria become dominant, rendering antibiotics less effective (Tello et al. 2012, Serwecińska 2020).

## CHAPTER 3: PUBLICATION

---

---

*At the time of submission, this manuscript was submitted to: Journal of Essential Oil Bearing Plants, Taylor & Francis, July 2024.*

### **Unveiling the Potential of Essential Oils against Bacterial Resistance**

K. Naidoo<sup>1</sup>, S. F. van Vuuren<sup>1</sup>, A. Orchard<sup>1</sup>

#### **Affiliation**

<sup>1</sup>University of the Witwatersrand, Faculty of Health Sciences, Department of Pharmacy and Pharmacology, 7 York Road, Parktown 2193, South Africa

#### **Correspondence**

Ané Orchard, Department of Pharmacy and Pharmacology, Faculty of Health Sciences,  
University of the Witwatersrand, 7 York Road, Parktown 2193, South Africa

Email: ane.orchard@wits.ac.za

## Abstract

Essential oils (EOs), derived from aromatic plants, have been widely studied and used as antimicrobials due to their broad-spectrum activity. However, little is known about the impact EOs will have on resistance emergence due to exposure in relation to resistance mechanisms. This study aimed to investigate the minimum inhibitory activity of 23 essential oils (EOs) and thereafter investigate the resistance development against five EOs, with varying inhibitory activities. Anti-quorum sensing (anti-QS) and biofilm inhibition assays were additionally performed to investigate resistant mechanisms. In the current study, 12 EOs ( $\pm 52.00\%$ ) exhibited noteworthy ( $\leq 1.00$  mg/mL) inhibition against multi-drug-resistant pathogens via the microdilution assay. The percentage inhibition of violacein was assessed to determine anti-QS activity. Biofilm inhibition was evaluated using the crystal violet assay. Resistance induction studies determined patterns in the MIC values of the five EOs, over 20 passages against *S. aureus* ATCC 6538, due to repeated exposure of the culture to sub-inhibitory concentrations. *Origanum vulgare* and *Thymus vulgaris* EOs showed notable bacterial inhibition (1.00 mg/mL), anti-QS, and biofilm inhibition activity (70.00% inhibition). *Staphylococcus aureus* ATCC 6538 exhibited heteroresistance to antibiotics post-EO exposure, however, no long-term resistance to EOs was observed; instead, temporary and significant MIC changes ( $p$ -value = 0.00 - 0.02), after which the MIC value reverted in the next passage. These results underscore the potential of EOs in combating the ESKAPE pathogens and offer promising solutions in addressing the global issue of antimicrobial resistance (AMR). The study substantiates the use of EO as an alternative antimicrobial based on its effectiveness against multi-drug resistant pathogens. Furthermore, based on the five EOs tested, it was highlighted that EOs exhibiting noteworthy inhibitory activity are also associated with anti-QS and biofilm inhibition activities, alongside the ability to sustain MICs upon repeated exposure. Thus, contributing to the overall understanding of EOs in combatting resistance.

**Keywords**

Resistance induction, ESKAPE pathogens, essential oils, quorum sensing, biofilm

**Short running head**

Essential oils against bacterial resistance

## Introduction

Essential oils (EOs), derived from aromatic plants, have a wide range of applications, including use in food, cosmetics, and medicine (Naeem et al. 2018). Essential oils have demonstrated antimicrobial activity against planktonic bacteria (free-living) in a wide array of studies. For many of these oils, the antimicrobial activity is against both Gram-positive and Gram-negative planktonic bacteria, thereby exerting a broad-spectrum antimicrobial activity, including against the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter*) pathogens (de Rapper et al. 2013, Hristova et al. 2013, SAMF 2016, Orchard et al. 2017). The ESKAPE pathogens have been highlighted by the World Health Organization (WHO) as being the most problematic micro-organisms leading to difficult-to-treat infections. The challenge of bacterial resistance to antibiotics is highlighted in several studies and it is well-known that drug-resistant pathogens have led to higher mortality rates in recent years (Ahmed et al. 2024, Sartorius et al. 2024). Despite the promising antimicrobial effects of EOs, research regarding the role of EOs in combating resistance remains largely neglected. This knowledge gap poses a significant challenge in leveraging EOs efficiently within the medical field to combat resistance. In contrast, the food industry has extensively covered this area, stressing the urgency to comprehensively expand research efforts to explain the relationship between EOs and resistance.

Quorum sensing (QS) is a communication mechanism utilised by micro-organisms to regulate gene expression based on cell density which impacts the development and spread of antimicrobial resistance (AMR) (Yu et al. 2020). Essential oils including *Origanum vulgare*, *Foeniculum vulgare*, *Melaleuca alternifolia*, *Syzygium aromaticum* and *Thymus vulgaris* have been reported to hinder this communication thus exhibiting anti-QS potential (Noumi et al.

2018, Cáceres et al. 2020, Moradi et al. 2020, Reichling 2020, D'Aquila et al. 2023). While studies focusing on this are evident, it is an area in research that warrants further investigation.

Biofilms are dense microbial communities encased in a protective matrix, facilitating resistance to antimicrobial agents. Micro-organisms in a biofilm can, in fact, be up to 1000-fold more resistant to antibiotics than planktonic bacteria and can efficiently evade the immune system (Oppenheimer-Shaanan et al. 2013). Several studies have demonstrated that EOs have the potential to inhibit and eradicate biofilm formation (Bazargani and Rohloff 2016, Cáceres et al. 2020, El-Tarabily et al. 2021). The review conducted by El-Tarabily et al. (2021) emphasized the potential of EOs as biofilm inhibitors, which could prove beneficial in combatting AMR. Biofilms and QS both contribute toward an increase in AMR as this process allows populations of micro-organisms to interact and act in a synchronized manner (Greenberg 2003, Li and Tian 2012, Kerekes et al. 2019). Essential oils have been identified as displaying anti-QS and biofilm inhibitory properties, thereby potentially contributing to the reduction of resistance development (Mahumane 2016, Oh et al. 2017, Camele et al. 2019, Kerekes et al. 2019).

Previous studies demonstrate resistance developed through biofilm formation, to sanitizers and other antimicrobial agents as a consequence of repeated exposure to these substances (Pekmezovic et al. 2016, Kerekes et al. 2019, Willems et al. 2016). Essential oils are widely used in skin products as well as hand sanitizers at low concentrations. The use of antimicrobials at sub-inhibitory concentrations promotes the development of resistance (Chakraborty et al. 2022, Sanchez-Cid et al. 2022). There is evidence demonstrating EOs' potential to withstand resistance, which is said to be due to the multi-compound nature of EOs (Fimbres-García et al. 2022, Kong et al. 2022, Panda et al. 2022). This allows for multi-target attacks, making it difficult for pathogens to adapt in a manner that prevents antimicrobial activity at multiple target sites (Becerril et al. 2012, Yap et al. 2014). However, Bermúdez-

Capdevila et al. (2022) reported that the repeated exposure of various serotypes of *Listeria monocytogenes* to sub-inhibitory levels of *Cinnamomum cassia* Blume over three days resulted in significant changes ( $p$ -value = 0.013) in the minimum inhibitory concentration (MIC) values. Over the three days, the concentration of the EO that was exposed to the pathogen was increased daily, to mimic an accumulative effect, however, it was still at sub-inhibitory concentrations. Furthermore, this repeated exposure caused alterations in antibiotic susceptibility profiles, increasing the susceptibility of different serotypes of *Listeria monocytogenes* to three conventional antibiotics (ciprofloxacin, cefepime, and enrofloxacin), while it became resistant against cefotaxime and cefepime (Bermúdez-Capdevila et al. 2022). These findings highlight the variable effects of antibiotics on bacteria exposed to sub-inhibitory concentrations of essential oils.

The induction of resistance against EOs requires consideration, particularly in light of evidence indicating that sub-lethal exposure to *M. alternifolia* leads to a decreased bacterial susceptibility to this oil (Nelson 2000). Furthermore, the sub-lethal exposure to the EO affected how the bacteria responded to the antibiotics, where a lowered susceptibility to the conventional antibiotics was observed (McMahon et al. 2008, Nelson 2000). Currently, available studies on resistance induction to EOs are limited to a few pathogens and EOs, with the main focus being on food pathogens (Becerril et al. 2012). Further investigation is needed on EOs that have shown notable inhibition against multi-drug resistant pathogens. It is not known whether the resistance that occurred may be dependent on the overall antimicrobial activity of the EO, thus it is not known whether an EO with stronger antimicrobial activity would be better suited to withstanding resistance.

It is not yet clear whether the induced resistance may be related to resistance pathways such as QS and biofilm formation. By incorporating all four assays (MIC, anti-QS, biofilm inhibition and induction resistance), a more comprehensive understanding of the relationship

between EOs and resistance mechanisms, particularly the induction of resistance, can be established. Additionally, it is important to understand whether EO will affect the activity of other antimicrobials as previously demonstrated by Bermúdez-Capdevila et al. (2022). This becomes even more important when considering the frequent use of products containing low concentrations of EOs. Thus, this study aimed to further investigate the effect of five specific EOs on resistant mechanisms and their potential to induce resistance due to repeated exposure at sub-inhibitory concentrations.

## **Materials and methods**

### ***Essential oil selection and chemistry***

The selection of EOs was based on previous studies investigating EOs against one or more of the ESKAPE pathogens. However, the main focus was given to EOs that reported notable antimicrobial activity from our previous study (Orchard et al. 2017) (Supplementary Table S1). In total, 23 EOs were selected for the initial antimicrobial screening. The EOs were procured from international flavour and fragrance industries such as Prana Monde, Essentia, Subtle Energies, and Scatters Oils<sup>cc</sup> (Gauteng, South Africa). The chemical analysis of several EOs was conducted in 2017 by Orchard et al. (2017) using Gas chromatography-mass spectrometry (GC-MS) or provided as a certificate of analysis from the supplier, with the main constituents listed (Supplementary Table S2). All essential oils were stored at -20°C and sub-aliquoted into amber bottles for use when needed. All essential oils were used before their expiry date.

### ***Preparation of cultures***

All micro-organisms were cultured in Tryptone Soya Broth (TSB) (Oxoid) and on Tryptone Soya Agar (TSA) (Oxoid), and incubated at 37°C for 24 hrs. The original stock

culture was streaked onto TSA, to ensure purity by uniform colony morphology. All cultures were maintained and stored at 4°C. The reference and antibiotic-resistant strains used in this study comprise of the American Type Culture Collection (ATCC) strains (Davies Diagnostics Johannesburg, South Africa) or Deutsche Sammlung von Mikroorganismen (DSM) (Leibniz Institute, Braunschweig, Germany). The reference strains included *Enterococcus faecium* ATCC 27270, *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* ATCC 13883, *Acinetobacter baumannii* ATCC 19606, *Pseudomonas aeruginosa* ATCC 27853, *Enterobacter aerogenes* ATCC 13048. The resistant ATCC strains included *S. aureus* ATCC 6538, methicillin-resistant *S. aureus* (MRSA) ATCC 43300 and gentamicin and methicillin-resistant *S. aureus* (GMRSA) ATCC 33591. The clinical strains were sourced from the National Health Laboratory Service (NHLS) Infection Control and Microbiology Laboratory (University of Witwatersrand).

### ***Antimicrobial susceptibility***

Antimicrobial susceptibility testing was performed for all strains against a wide range of conventional antibiotics, using the disc diffusion method (CLSI 2020). Antimicrobial susceptibility testing is of utmost importance in order to determine the resistant profiles of the strains employed in this study, particularly for the purpose of classifying clinical strains as either resistant or susceptible. The antibiotics tested cover a range of classes critical for combating bacterial infections. They include agents that disrupt bacterial protein synthesis, inhibit cell wall synthesis, interfere with DNA replication and repair, and inhibit folic acid synthesis.

### ***Minimum inhibitory activity***

The broth microdilution method was used to quantify the inhibitory activity of the EOs (CLSI 2020). The EOs were diluted to a stock concentration of 32.00 mg/mL using absolute acetone (Merck). Positive controls (ciprofloxacin 0.01 mg/mL) and negative controls (water diluted with acetone to attain a concentration of 32.00 mg/mL) were included. A culture control was included to monitor the viability of each bacterial strain. A two-fold dilution was undertaken initially, with the EO (100  $\mu$ L) and TSB (100  $\mu$ L), to achieve concentrations ranging from 0.06 to 8.00 mg/mL, and the antibiotic concentration ranging from 0.20 to 2.50  $\mu$ g/mL. Culture suspensions (0.50 McFarland turbidity standard) were made and subsequently diluted to achieve an approximate concentration of  $1 \times 10^6$  CFU/mL. This was then added to all the wells at a volume of 100  $\mu$ L. Each microtiter plate was sealed with a sterile adhesive sealing film, to prevent loss of the samples due to evaporation. All bacterial strains were incubated at 37°C for 24 hrs. After the 24-hour incubation, 40  $\mu$ L *p*-iodonitrotetrazolium violet solution (INT) (0.04 mg/mL) was added as a growth indicator. The results were recorded after six hours, and the lowest dilution with no color change was interpreted as the MIC for that EO. A MIC value of  $\leq 1.00$  mg/mL was considered noteworthy for the EOs (Orchard et al. 2017b). The classification of these oils for this study was based on the distribution of data obtained from Orchard and van Vuuren (2017). Through the screening process, five EOs were selected based on their representation of varying inhibitory activity against the ESKAPE pathogens, ranging from noteworthy (MIC  $\leq 1.00$ ) to moderate (MIC = 1.00 - 3.00 mg/mL) and poor ( $\geq 3.00$  mg/mL) inhibition. These selected oils were further investigated in the anti-QS, biofilm inhibition and induction of resistance studies.

### ***Anti-quorum sensing***

The broth macrodilution method, as described by Mahumane (2016), was used to screen for anti-QS activity of the five selected essential oils. This method allows for both the qualitative (color change) and quantitative (percentage inhibition) determination of the ability of the EOs to inhibit QS. Essential oils were added to 5.00 mL of Luria-Bertani (LB) broth (Oxoid), resulting in concentrations ranging from 0.03 to 8.00 mg/mL. *Chromobacterium violaceum* (ATCC 12472) suspension (100.00 µl) was added to each test tube and then incubated for 24 hrs at 30°C and agitated at 140 rpm, in an orbital shaker incubator (Labcon). The presence of anti-QS activity was observed by the presence of a purple pigment. After centrifugation, the supernatant was analyzed to determine the violacein content by recording the absorbance at OD595 nm, using a FilterMax F5 multimode microplate reader (Molecular Devices). The percentage of violacein inhibition was calculated according to Equation 1.

Equation 1:

$$\text{Percentage violacein production} = \frac{\text{control Optical density (OD)} - \text{OD experimental}}{\text{control OD}} \times 100$$

The minimum QS inhibitory concentration (MQSIC) is defined as the concentration at which the percentage inhibition is  $\geq 70.00\%$ . Percentage inhibition of  $\geq 70.00\%$  is indicative of noteworthy anti-QS activity, based on herd immunity theory (Jansen et al., 2021). The minimum quorum sensing bactericidal concentration (MQSBC) is defined as the first concentration that prevents the growth of *C. violaceum*, when streaked out onto an agar plate.

### ***Biofilm inhibition***

The method as described by Singh (2022) was used for the biofilm inhibition studies, with additional modifications. Essential oils (100 µL) were added into the wells of a 96-well

microtiter plate. A 100  $\mu\text{L}$  of the standardized solution containing approximately  $1 \times 10^6$  CFU/mL of the culture, *S. aureus* ATCC 6538, was added into the wells and incubated at  $37^\circ\text{C}$  for 24 hrs. It was reported that *S. aureus* ATCC 6538 has strong biofilm-forming properties hence it was used in this assay (Abbas et al. 2019, Koçak et al. 2022). Positive controls (ciprofloxacin, gentamicin, and erythromycin) and a negative control (water in acetone) were included. The crystal violet assay was undertaken. This comprised of washing the incubated plates with sterile water, which was done in triplicate, to remove planktonic bacteria. The microtiter plates were dried at  $50^\circ\text{C}$  for 30 min, after which the wells were stained with 150  $\mu\text{L}$  of 0.10% crystal violet, and kept at room temperature for 15 min, followed by washing with sterile water to remove excess dye. To assess biofilm formation, 150  $\mu\text{L}$  of acetic acid (33.00%) was added to dissolve the stained biofilm. Absorbance was determined at 595 nm using a microplate reader (Universal microplate reader ELX 800). The mean absorbance of the EO was determined, and percentage inhibition was calculated using Equation 2.

Equation 2:

$$\text{Percentage biofilm inhibition} = \frac{\text{OD culture control} - \text{OD experimental}}{\text{OD culture control}} \times 100$$

### ***Resistance induction***

The method for induction of resistance was taken from Hammer et al. (2012), with modifications. The inoculum was prepared to achieve an approximate concentration of  $1 \times 10^6$  CFU/mL of *S. aureus* ATCC 6538. This specific strain is commonly employed in studies on antibiotic susceptibility research on infectious diseases, and as a means of quality control (Koçak et al. 2022). The broth microdilution method was used to determine the initial MIC of the five EOs against *S. aureus* (CLSI 2020). Next, the MIC well, two concentrations higher, and two concentrations lower was streaked onto agar plates and incubated at  $37^\circ\text{C}$  for 24 hrs.

The lowest concentration that exhibited growth was used to subculture the next isolate. These isolates were incubated for 3 hrs, before the microdilution method, allowing for the micro-organism to be exposed to the EO during the exponential phase of growth (Locke et al. 2009, Foster 2017). The broth microdilution method was repeated, however, using the new sub-cultured isolate. With every subsequent broth microdilution, *S. aureus* ATCC 6538, the pathogen was exposed to sub-inhibitory concentrations of the EO. This sequence was repeated for a total of 20 passages. Resistance was defined as an increase in the MIC value by more than two serial dilutions between two subsequent passages, with this increase in MIC being sustained throughout the passages, thereafter. An increase or decrease in the MIC by two serial dilutions was considered to be a result of natural biological and methodological variation.

### ***Statistical analysis***

All assays in this study were performed in duplicate on three different days (therefore n=6), this excluded the resistance induction assay. The results from the initial three assays were averaged and the standard deviation (SD) was calculated. For the resistance induction assay, passages were repeated when changes of more than two serial dilutions in the MIC were observed, to verify the results. The SD across all 20 passages was calculated, and a SD of  $\geq 1.00$  was considered to be a significant variance. Student T-test was used to determine whether there was a statistically significant shift in the MIC for each oil. In all instances, a significance threshold of  $p < 0.05$  was established.

## **Results and Discussion**

### ***Essential oil chemistry***

The chemical composition of each of the oils was analyzed and an abridged set of results detailing the identified main compounds is given in Supplementary data Table S2.

Although this study does not focus on individual compounds, the composition of EOs significantly influences their antimicrobial activity (Burt 2004). Therefore, stating the composition is important. The comparison of chemotypes becomes crucial when specific EOs are identified as potential antimicrobial agents.

### ***Antibiotic susceptibility of cultures***

The results of the antibiotic susceptibility test revealed that resistant and susceptible clinical strains, in addition to the reference strains, were tested in the study (Table 1). The primary objective of this assay was to acquire the resistance profiles of each strain for comparison with the susceptibility of the EOs.

### ***Minimum inhibitory activity***

Table 2 displays the mean MIC values of 23 EOs tested against each of the 18 bacterial ESKAPE strains. *Origanum vulgare* and *T. vulgaris* demonstrated notable and broad-spectrum inhibitory activity with an average MIC value of 0.78 mg/mL and 0.92 mg/mL respectively. *Matricaria recutita* and *C. myrrha*, demonstrated the least minimum inhibitory activity against the ESKAPE pathogens, with only one and two cases of noteworthy inhibition, both against a clinical strain of *P. aeruginosa* (Resistant Clinical 2) and *C. myrrha* against *S. aureus* ATCC 25923. *Santalum austrocaledonicum* and *Vetiveria zizanioides* were the only two EOs that had noteworthy inhibitory activity against both strains of *E. faecium*. Furthermore, both oils exhibited the greatest inhibitory activity against the Gram-positive bacteria, *E. faecium* and *S. aureus*. Ten ( $\pm$  43.00%) EOs (*C. carvi*, *C. verum*, *C. zeylanicum*, *C. martini*, *F. dulce*, *O. tenuiflorum*, *O. vulgare*, *R. damascena*, *S. aromaticum* and *T. vulgaris*) exhibited noteworthy inhibition against the multi-drug resistant strain *A. baumannii*, which was resistant to all antibiotics tested including meropenem (Table 1).

Table 1. Antimicrobial susceptibility testing

| Antibiotic                    | <i>E. faecium</i> |                    | <i>S. aureus</i> |           |            |                    |                      |            | <i>K. pneumoniae</i> |            |            | <i>A. baumannii</i> |                    | <i>P. aeruginosa</i> |                      |                      | <i>E. aerogenes</i> |                    |
|-------------------------------|-------------------|--------------------|------------------|-----------|------------|--------------------|----------------------|------------|----------------------|------------|------------|---------------------|--------------------|----------------------|----------------------|----------------------|---------------------|--------------------|
|                               | ATCC 27270        | Resistant Clinical | ATCC 25923       | ATCC 6538 | ATCC 43300 | Resistant Clinical | Susceptible Clinical | ATCC 33592 | ATCC 13883           | Clinical 1 | Clinical 2 | ATCC 19606          | Resistant Clinical | ATCC 27853           | Resistant Clinical 1 | Resistant Clinical 2 | ATCC 13048          | Resistant Clinical |
| Amikacin                      | S* <sup>1</sup>   | R                  | S                | S         | S          | R                  | S                    | S          | S                    | S          | S          | S                   | I                  | S                    | S                    | S                    | S                   | S                  |
| Amoxicillin                   | R* <sup>2</sup>   | R                  | S                | S         | I          | R                  | I                    | I          | R                    | I          | S          | R                   | R                  | R                    | R                    | R                    | R                   | R                  |
| Amoxicillin – Clavulanic acid | S                 | R                  | S                | S         | I          | S                  | I                    | S          | R                    | I          | S          | S                   | R                  | R                    | R                    | R                    | R                   | R                  |
| Ampicillin                    | S                 | R                  | S                | S         | I          | R                  | S                    | S          | R                    | S          | S          | R                   | R                  | R                    | R                    | R                    | R                   | R                  |
| Ceftriaxone                   | S                 | R                  | S                | S         | S          | R                  | S                    | S          | S                    | S          | S          | I                   | R                  | R                    | R                    | R                    | S                   | R                  |
| Cephalexin                    | R                 | R                  | S                | S         | S          | R                  | S                    | S          | S                    | I          | S          | R                   | R                  | R                    | R                    | R                    | R                   | R                  |
| Cephalothin                   | R                 | R                  | S                | S         | S          | R                  | S                    | S          | S                    | S          | S          | R                   | R                  | R                    | R                    | R                    | R                   | R                  |
| Chloramphenicol               | I* <sup>3</sup>   | R                  | S                | S         | S          | R                  | S                    | S          | S                    | S          | S          | S                   | R                  | S                    | R                    | R                    | S                   | R                  |
| Ciprofloxacin                 | S                 | R                  | S                | S         | S          | R                  | S                    | S          | S                    | S          | S          | S                   | R                  | S                    | S                    | S                    | S                   | S                  |
| Clindamycin                   | S                 | R                  | S                | S         | S          | S                  | S                    | S          | R                    | R          | R          | R                   | R                  | R                    | R                    | R                    | R                   | R                  |
| Erythromycin                  | R                 | R                  | S                | S         | S          | S                  | S                    | S          | R                    | R          | R          | R                   | R                  | R                    | R                    | R                    | R                   | R                  |
| Fosfomycin                    | R                 | R                  | S                | S         | S          | I                  | S                    | S          | R                    | S          | S          | I                   | R                  | R                    | R                    | I                    | I                   | S                  |
| Gentamicin                    | S                 | R                  | S                | I         | I          | R                  | I                    | S          | S                    | S          | I          | S                   | R                  | S                    | I                    | I                    | S                   | S                  |
| Meropenem                     | S                 | R                  | S                | S         | S          | S                  | S                    | S          | S                    | S          | S          | S                   | R                  | S                    | S                    | S                    | S                   | S                  |
| Neomycin                      | R                 | R                  | S                | I         | S          | R                  | S                    | S          | S                    | I          | I          | R                   | R                  | R                    | R                    | R                    | R                   | I                  |
| Penicillin                    | R                 | R                  | S                | S         | I          | R                  | I                    | I          | R                    | R          | R          | R                   | R                  | R                    | R                    | R                    | R                   | R                  |
| Piperacillin-Tazobactam       | S                 | R                  | S                | S         | I          | R                  | S                    | S          | S                    | S          | S          | S                   | R                  | S                    | S                    | S                    | S                   | R                  |
| Sulfamethoxazole-Trimethoprim | I                 | R                  | S                | S         | S          | R                  | I                    | S          | I                    | S          | S          | S                   | R                  | S                    | R                    | R                    | I                   | R                  |
| Tetracycline                  | S                 | R                  | S                | S         | I          | R                  | S                    | S          | S                    | I          | I          | S                   | R                  | I                    | R                    | R                    | I                   | R                  |
| Vancomycin                    | S                 | I                  | S                | I         | I          | S                  | S                    | I          | R                    | R          | R          | R                   | R                  | R                    | R                    | R                    | R                   | R                  |

\*<sup>1</sup>Susceptible = Microbe is “susceptible” to the antibiotic and can be used for testing, \*<sup>2</sup>R - Resistant = Microbe is resistant to antibiotics and

\*<sup>3</sup>I – Intermediate = Microbe is partially “susceptible” to the antibiotic, but MIC values may exceed breakpoints outlined in CLSI and EUCAST.

(CLSI 2020, EUCAST 2022).

Table 2. Minimum inhibitory activity (mg/mL ( $\pm$ SD)) of essential oils (n=3)

| Essential oil         | <i>E. faecium</i>      |                       | <i>S. aureus</i>             |                              |                              |                              |                              |                              | <i>K. pneumoniae</i>         |                              |                              | <i>A. baumannii</i>   |                              | <i>P. aeruginosa</i>         |                              |                              | <i>E. aerogenes</i>          |                              | Average |
|-----------------------|------------------------|-----------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|-----------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|---------|
|                       | ATCC 27270             | Resistant Clinical    | ATCC 25923                   | ATCC 6538                    | ATCC 43300                   | Resistant Clinical           | Susceptible Clinical         | ATCC 33592                   | ATCC 13883                   | Clinical 1                   | Clinical 2                   | ATCC 19606            | Resistant Clinical           | ATCC 27853                   | Resistant Clinical 1         | Resistant Clinical 2         | ATCC 13048                   | Resistant Clinical           |         |
| <i>A. sativum</i>     | >8.00<br>( $\pm$ 0.00) | 2.00<br>( $\pm$ 0.00) | 3.20<br>( $\pm$ 1.10)        | 4.00<br>( $\pm$ 0.00)        | 2.00<br>( $\pm$ 0.00)        | 2.00<br>( $\pm$ 0.00)        | 3.00<br>( $\pm$ 0.00)        | 2.67<br>( $\pm$ 1.15)        | 2.00<br>( $\pm$ 0.00)        | 4.00<br>( $\pm$ 0.00)        | 2.40<br>( $\pm$ 2.42)        | 2.00<br>( $\pm$ 1.22) | 2.00<br>( $\pm$ 1.73)        | 2.00<br>( $\pm$ 0.00)        | <b>0.13</b><br>( $\pm$ 0.00) | <b>0.25</b><br>( $\pm$ 0.00) | 3.00<br>( $\pm$ 1.41)        | 3.00<br>( $\pm$ 1.41)        | 2.41    |
| <i>C. carvi</i>       | 4.00<br>( $\pm$ 0.00)  | 2.00<br>( $\pm$ 0.00) | 1.33<br>( $\pm$ 0.52)        | 2.00<br>( $\pm$ 0.00)        | <b>1.00</b><br>( $\pm$ 0.00) | 2.00<br>( $\pm$ 0.00)        | 2.00<br>( $\pm$ 0.00)        | 2.00<br>( $\pm$ 0.00)        | 1.60<br>( $\pm$ 1.10)        | 1.50<br>( $\pm$ 0.58)        | <b>1.00</b><br>( $\pm$ 0.00) | 1.67<br>( $\pm$ 0.52) | <b>1.00</b><br>( $\pm$ 0.00) | 2.00<br>( $\pm$ 0.00)        | <b>1.00</b><br>( $\pm$ 0.00) | <b>1.00</b><br>( $\pm$ 0.00) | <b>1.00</b><br>( $\pm$ 0.00) | <b>1.00</b><br>( $\pm$ 0.00) | 1.62    |
| <i>C. verum</i>       | 2.00<br>( $\pm$ 0.00)  | 2.00<br>( $\pm$ 0.00) | 3.20<br>( $\pm$ 1.10)        | 2.00<br>( $\pm$ 0.00)        | <b>0.68</b><br>( $\pm$ 0.31) | 2.00<br>( $\pm$ 0.00)        | <b>1.00</b><br>( $\pm$ 0.00) | <b>1.00</b><br>( $\pm$ 0.00) | <b>1.00</b><br>( $\pm$ 0.00) | <b>1.00</b><br>( $\pm$ 0.00) | <b>1.00</b><br>( $\pm$ 0.00) | 1.80<br>( $\pm$ 0.92) | <b>0.59</b><br>( $\pm$ 0.35) | <b>1.00</b><br>( $\pm$ 0.00) | <b>1.00</b><br>( $\pm$ 0.00) | 3.00<br>( $\pm$ 1.41)        | <b>1.00</b><br>( $\pm$ 0.00) | <b>1.00</b><br>( $\pm$ 0.00) | 1.46    |
| <i>C. zeylanicum</i>  | 2.00<br>( $\pm$ 0.00)  | 2.00<br>( $\pm$ 0.00) | 1.60<br>( $\pm$ 0.55)        | 2.00<br>( $\pm$ 0.00)        | <b>0.64</b><br>( $\pm$ 0.35) | 1.25<br>( $\pm$ 0.50)        | <b>1.00</b><br>( $\pm$ 0.00) | <b>1.00</b><br>( $\pm$ 0.00) | 1.13<br>( $\pm$ 0.35)        | <b>1.00</b><br>( $\pm$ 0.00) | <b>1.00</b><br>( $\pm$ 0.00) | 1.30<br>( $\pm$ 0.63) | <b>0.58</b><br>( $\pm$ 0.34) | <b>1.00</b><br>( $\pm$ 0.00) | <b>1.00</b><br>( $\pm$ 0.38) | 2.00<br>( $\pm$ 0.00)        | <b>1.00</b><br>( $\pm$ 0.00) | <b>1.00</b><br>( $\pm$ 0.00) | 1.25    |
| <i>C. myrrha</i>      | >8.00<br>( $\pm$ 0.00) | 3.00<br>( $\pm$ 1.41) | <b>1.00</b><br>( $\pm$ 1.64) | 2.00<br>( $\pm$ 0.00)        | 5.33<br>( $\pm$ 2.31)        | 3.00<br>( $\pm$ 1.41)        | 2.00<br>( $\pm$ 0.00)        | 8.00<br>( $\pm$ 0.00)        | 2.00<br>( $\pm$ 0.00)        | >8.00<br>( $\pm$ 0.00)       | 2.00<br>( $\pm$ 0.00)        | 5.20<br>( $\pm$ 3.83) | >8.00<br>( $\pm$ 0.00)       | 2.00<br>( $\pm$ 0.00)        | <b>1.00</b><br>( $\pm$ 0.00) | >8.00<br>( $\pm$ 0.00)       | 2.00<br>( $\pm$ 0.00)        | 4.00<br>( $\pm$ 0.00)        | 3.04    |
| <i>C. citratus</i>    | 2.00<br>( $\pm$ 0.00)  | 2.00<br>( $\pm$ 0.00) | <b>1.00</b><br>( $\pm$ 0.28) | <b>0.80</b><br>( $\pm$ 0.57) | 2.67<br>( $\pm$ 1.15)        | <b>1.00</b><br>( $\pm$ 0.00) | <b>0.50</b><br>( $\pm$ 0.00) | <b>0.88</b><br>( $\pm$ 0.25) | 2.00<br>( $\pm$ 0.00)        | 2.00<br>( $\pm$ 0.00)        | 3.00<br>( $\pm$ 1.41)        | 1.60<br>( $\pm$ 0.55) | 2.00<br>( $\pm$ 0.00)        | 3.00<br>( $\pm$ 1.41)        | <b>1.00</b><br>( $\pm$ 0.00) | >8.00<br>( $\pm$ 0.00)       | 2.00<br>( $\pm$ 0.00)        | 3.00<br>( $\pm$ 1.41)        | 1.79    |
| <i>C. martinii</i>    | 2.00<br>( $\pm$ 0.00)  | 2.00<br>( $\pm$ 0.00) | 1.60<br>( $\pm$ 0.55)        | 2.00<br>( $\pm$ 0.00)        | 2.00<br>( $\pm$ 0.00)        | 2.00<br>( $\pm$ 0.00)        | <b>1.00</b><br>( $\pm$ 0.00) | <b>1.00</b><br>( $\pm$ 0.00) | 2.00<br>( $\pm$ 0.00)        | 2.00<br>( $\pm$ 0.00)        | 3.00<br>( $\pm$ 1.41)        | 1.60<br>( $\pm$ 0.55) | <b>0.80</b><br>( $\pm$ 0.33) | 2.00<br>( $\pm$ 0.00)        | <b>1.00</b><br>( $\pm$ 0.00) | >8.00<br>( $\pm$ 0.00)       | 2.00<br>( $\pm$ 0.00)        | 3.00<br>( $\pm$ 1.41)        | 1.82    |
| <i>F. galbaniflua</i> | 8.00<br>( $\pm$ 0.00)  | 4.00<br>( $\pm$ 0.00) | 3.50<br>( $\pm$ 0.93)        | 3.33<br>( $\pm$ 1.15)        | 2.00<br>( $\pm$ 0.00)        | 2.00<br>( $\pm$ 0.00)        | 2.00<br>( $\pm$ 0.00)        | 2.00<br>( $\pm$ 0.00)        | 4.00<br>( $\pm$ 0.00)        | 2.00<br>( $\pm$ 0.00)        | 2.20<br>( $\pm$ 1.10)        | 3.20<br>( $\pm$ 1.10) | 1.33<br>( $\pm$ 0.58)        | 3.00<br>( $\pm$ 1.41)        | <b>1.00</b><br>( $\pm$ 0.00) | 2.00<br>( $\pm$ 0.00)        | 6.00<br>( $\pm$ 2.83)        | 4.00<br>( $\pm$ 0.00)        | 3.09    |

| Essential oil          | <i>E. faecium</i> |                        | <i>S. aureus</i>       |                        |                        |                    |                        |                        | <i>K. pneumoniae</i>   |                        |                        | <i>A. baumannii</i>    |                        | <i>P. aeruginosa</i>   |                        |                        | <i>E. aerogenes</i>    |                        | Average     |
|------------------------|-------------------|------------------------|------------------------|------------------------|------------------------|--------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|-------------|
|                        | ATCC 27270        | Resistant Clinical     | ATCC 25923             | ATCC 6538              | ATCC 43300             | Resistant Clinical | Susceptible Clinical   | ATCC 33592             | ATCC 13883             | Clinical 1             | Clinical 2             | ATCC 19606             | Resistant Clinical     | ATCC 27853             | Resistant Clinical 1   | Resistant Clinical 2   | ATCC 13048             | Resistant Clinical     |             |
| <i>F. dulce</i>        | 4.00<br>(±0.00)   | 2.00<br>(±0.00)        | 2.00<br>(±0.00)        | 2.67<br>(±1.03)        | 2.00<br>(±0.00)        | 2.00<br>(±0.00)    | 2.00<br>(±0.00)        | 2.00<br>(±0.00)        | 1.60<br>(±1.10)        | 1.50<br>(±0.58)        | <b>1.00</b><br>(±0.00) | 1.67<br>(±0.52)        | <b>0.90</b><br>(±0.33) | 2.00<br>(±0.00)        | <b>1.00</b><br>(±0.00) | <b>1.00</b><br>(±0.00) | 1.25<br>(±0.50)        | <b>1.00</b><br>(±0.00) | 1.75        |
| <i>L. nobilis</i>      | 8.00<br>(±0.00)   | 4.00<br>(±0.00)        | 3.20<br>(±1.10)        | 4.00<br>(±0.00)        | 3.33<br>(±1.15)        | 2.00<br>(±0.00)    | 3.00<br>(±0.00)        | 4.00<br>(±0.00)        | 5.33<br>(±2.31)        | 2.40<br>(±1.52)        | 3.00<br>(±1.41)        | 3.20<br>(±1.10)        | 1.42<br>(±1.42)        | 2.00<br>(±0.00)        | <b>1.00</b><br>(±0.00) | 2.00<br>(±0.00)        | 2.00<br>(±0.00)        | 6.00<br>(±2.83)        | 3.33        |
| <i>L. angustifolia</i> | 8.00<br>(±0.00)   | 3.00<br>(±1.41)        | 3.20<br>(±1.10)        | 4.00<br>(±0.00)        | 2.67<br>(±1.15)        | 2.00<br>(±0.00)    | 2.00<br>(±0.00)        | 4.00<br>(±0.00)        | 2.00<br>(±0.00)        | 2.67<br>(±1.15)        | 3.00<br>(±1.41)        | 2.40<br>(±0.89)        | 2.50<br>(±1.00)        | 4.00<br>(±0.00)        | <b>1.00</b><br>(±0.00) | 2.00<br>(±0.00)        | 2.00<br>(±0.00)        | 4.00<br>(±0.00)        | 3.02        |
| <i>L. cubeba</i>       | 2.00<br>(±0.00)   | 1.75<br>(±0.50)        | 1.60<br>(±0.55)        | 2.00<br>(±0.00)        | 2.00<br>(±0.00)        | 1.25<br>(±0.50)    | <b>0.88</b><br>(±0.00) | <b>0.88</b><br>(±0.25) | 2.00<br>(±0.00)        | 2.00<br>(±0.00)        | 2.00<br>(±0.00)        | 1.80<br>(±0.45)        | 2.00<br>(±0.00)        | 2.00<br>(±0.00)        | <b>1.00</b><br>(±0.00) | >8.00<br>(±0.00)       | 2.00<br>(±0.00)        | 4.00<br>(±0.00)        | 1.83        |
| <i>M. recutita</i>     | >8.00<br>(±0.00)  | 3.00<br>(±1.41)        | 3.20<br>(±1.10)        | 4.00<br>(±0.00)        | 4.00<br>(±0.00)        | 4.00<br>(±0.00)    | 4.00<br>(±0.00)        | 4.00<br>(±0.00)        | 2.00<br>(±0.00)        | 3.33<br>(±1.15)        | 4.00<br>(±0.00)        | 3.20<br>(±1.10)        | 4.00<br>(±0.00)        | 4.00<br>(±0.00)        | <b>1.00</b><br>(±0.00) | >8.00<br>(±0.00)       | 2.00<br>(±0.00)        | 4.00<br>(±0.00)        | 3.36        |
| <i>M. alternifolia</i> | >8.00<br>(±0.00)  | 3.00<br>(±1.41)        | 2.00<br>(±0.00)        | 5.33<br>(±2.31)        | 4.00<br>(±0.00)        | 3.20<br>(±1.10)    | 4.00<br>(±0.00)        | 4.00<br>(±0.00)        | 3.33<br>(±1.15)        | 2.40<br>(±1.52)        | 4.00<br>(±0.00)        | 2.57<br>(±0.98)        | 2.50<br>(±1.00)        | 3.00<br>(±1.41)        | <b>1.00</b><br>(±0.00) | >8.00<br>(±0.00)       | 2.00<br>(±0.00)        | 3.00<br>(±1.41)        | 3.08        |
| <i>O. tenuiflorum</i>  | 2.00<br>(±0.00)   | 2.00<br>(±0.00)        | 1.75<br>(±0.46)        | 2.00<br>(±0.00)        | 2.00<br>(±0.00)        | 2.00<br>(±0.00)    | <b>0.75</b><br>(±0.00) | <b>0.92</b><br>(±0.20) | 1.40<br>(±0.55)        | 2.00<br>(±0.00)        | <b>1.00</b><br>(±0.00) | 1.40<br>(±0.82)        | <b>0.88</b><br>(±0.25) | 2.00<br>(±0.00)        | <b>1.00</b><br>(±0.00) | 2.00<br>(±0.00)        | 2.00<br>(±0.00)        | 1.25<br>(±0.50)        | 1.57        |
| <i>O. vulgare</i>      | 1.20<br>(±0.45)   | <b>1.00</b><br>(±0.00) | <b>0.61</b><br>(±0.31) | <b>0.83</b><br>(±0.25) | <b>0.67</b><br>(±0.28) | 1.25<br>(±0.50)    | <b>0.50</b><br>(±0.00) | <b>0.50</b><br>(±0.00) | <b>0.63</b><br>(±0.27) | <b>0.80</b><br>(±0.27) | <b>0.63</b><br>(±0.25) | <b>0.64</b><br>(±0.35) | <b>0.44</b><br>(±0.13) | <b>0.44</b><br>(±0.13) | <b>1.00</b><br>(±0.00) | 1.75<br>(±1.50)        | <b>0.64</b><br>(±0.27) | <b>0.46</b><br>(±0.10) | <b>0.78</b> |
| <i>P. graveolens</i>   | 4.00<br>(±0.00)   | 4.00<br>(±0.00)        | 1.71<br>(±0.93)        | 4.00<br>(±0.00)        | 2.00<br>(±0.00)        | 2.00<br>(±0.00)    | 2.00<br>(±0.00)        | 2.00<br>(±0.00)        | 2.00<br>(±0.00)        | 2.00<br>(±0.00)        | 3.00<br>(±1.41)        | 1.60<br>(±0.55)        | 2.00<br>(±0.00)        | 2.00<br>(±0.00)        | <b>1.00</b><br>(±0.00) | <b>1.00</b><br>(±0.00) | 2.00<br>(±0.00)        | 3.00<br>(±1.41)        | 2.30        |
| <i>P. patchouli</i>    | 2.00<br>(±0.00)   | 4.00<br>(±0.00)        | 1.50<br>(±0.71)        | 2.00<br>(±0.00)        | 4.00<br>(±0.00)        | 2.00<br>(±0.00)    | <b>0.35</b><br>(±0.00) | <b>0.59</b><br>(±0.35) | 3.33<br>(±1.15)        | 2.00<br>(±0.00)        | 3.00<br>(±1.41)        | 2.80<br>(±1.64)        | 2.00<br>(±0.00)        | 3.00<br>(±1.41)        | <b>1.00</b><br>(±0.00) | <b>1.00</b><br>(±0.00) | 2.00<br>(±0.00)        | 2.00<br>(±0.00)        | 2.14        |

| Essential oil              | <i>E. faecium</i>      |                        | <i>S. aureus</i>       |                        |                        |                        |                        |                        | <i>K. pneumoniae</i>   |                        |                        | <i>A. baumannii</i>    |                        | <i>P. aeruginosa</i>   |                        |                        | <i>E. aerogenes</i>    |                        | Average     |
|----------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|-------------|
|                            | ATCC 27270             | Resistant Clinical     | ATCC 25923             | ATCC 6538              | ATCC 43300             | Resistant Clinical     | Susceptible Clinical   | ATCC 33592             | ATCC 13883             | Clinical 1             | Clinical 2             | ATCC 19606             | Resistant Clinical     | ATCC 27853             | Resistant Clinical 1   | Resistant Clinical 2   | ATCC 13048             | Resistant Clinical     |             |
| <i>R. damascena</i>        | 2.00<br>(±0.00)        | 1.50<br>(±0.58)        | <b>0.84</b><br>(±0.30) | <b>1.00</b><br>(±0.00) | <b>0.50</b><br>(±0.00) | 2.00<br>(±0.00)        | <b>1.00</b><br>(±0.00) | <b>1.00</b><br>(±0.00) | 2.00<br>(±0.00)        | 2.67<br>(±1.15)        | 2.67<br>(±1.15)        | 1.33<br>(±0.52)        | <b>1.00</b><br>(±0.00) | 2.00<br>(±0.00)        | <b>1.00</b><br>(±0.00) | <b>1.00</b><br>(±0.00) | 2.20<br>(±1.10)        | 3.00<br>(±1.41)        | 1.60        |
| <i>S.austrocaledonicum</i> | <b>0.31</b><br>(±0.13) | <b>0.19</b><br>(±0.07) | <b>0.35</b><br>(±0.19) | <b>0.20</b><br>(±0.12) | 2.67<br>(±1.15)        | <b>0.38</b><br>(±0.14) | <b>0.13</b><br>(±0.00) | <b>0.21</b><br>(±0.07) | 2.50<br>(±2.61)        | 2.00<br>(±0.00)        | 4.00<br>(±0.00)        | 2.00<br>(±0.00)        | 2.00<br>(±0.00)        | 2.00<br>(±0.00)        | <b>1.00</b><br>(±0.00) | 1.25<br>(±0.50)        | 3.00<br>(±1.41)        | 2.00<br>(±0.00)        | 1.45        |
| <i>S. aromaticum</i>       | 3.00<br>(±1.41)        | 2.00<br>(±0.00)        | 1.29<br>(±0.49)        | 2.00<br>(±0.00)        | <b>0.83</b><br>(±0.32) | 2.00<br>(±0.00)        | <b>1.00</b><br>(±0.00) | <b>1.00</b><br>(±0.39) | <b>1.00</b><br>(±0.00) | <b>1.00</b><br>(±0.00) | <b>1.00</b><br>(±0.00) | 1.14<br>(±0.63)        | <b>0.67</b><br>(±0.38) | <b>1.00</b><br>(±0.00) | <b>1.00</b><br>(±0.00) | <b>1.00</b><br>(±0.00) | <b>1.00</b><br>(±0.00) | 1.20<br>(±0.45)        | 1.28        |
| <i>T. vulgaris</i>         | 1.40<br>(±0.55)        | 1.25<br>(±0.50)        | <b>0.90</b><br>(±0.28) | <b>0.80</b><br>(±0.27) | <b>1.00</b><br>(±0.00) | <b>1.00</b><br>(±0.00) | <b>0.75</b><br>(±0.00) | <b>0.50</b><br>(±0.00) | <b>0.90</b><br>(±0.22) | <b>1.00</b><br>(±0.00) | <b>1.00</b><br>(±0.00) | <b>0.86</b><br>(±0.24) | <b>0.44</b><br>(±0.13) | <b>0.88</b><br>(±0.25) | <b>1.00</b><br>(±0.00) | 1.20<br>(±0.45)        | <b>1.00</b><br>(±0.00) | <b>0.63</b><br>(±0.25) | <b>0.92</b> |
| <i>V. zizanioides</i>      | <b>0.50</b><br>(±0.00) | <b>0.50</b><br>(±0.00) | <b>0.25</b><br>(±0.38) | <b>0.40</b><br>(±0.14) | <b>3.33</b><br>(±1.15) | <b>0.50</b><br>(±0.00) | <b>0.13</b><br>(±0.00) | <b>0.13</b><br>(±0.45) | 2.67<br>(±1.15)        | 2.67<br>(±1.15)        | 2.00<br>(±0.00)        | 1.50<br>(±1.24)        | 2.00<br>(±0.00)        | 2.00<br>(±0.00)        | <b>1.00</b><br>(±0.00) | 1.20<br>(±1.21)        | 4.00<br>(±0.00)        | <b>1.00</b><br>(±0.00) | 1.43        |
| Positive Control (ug/mL)   | 2.50<br>(±0.00)        | >2.50<br>(±0.00)       | <b>0.63</b><br>(±0.00) | <b>0.31</b><br>(±0.89) | <b>0.04</b><br>(±0.00) | <b>0.49</b><br>(±0.17) | <b>0.50</b><br>(±0.00) | <b>0.63</b><br>(±0.00) | <b>0.07</b><br>(±0.02) | <b>0.04</b><br>(±0.00) | <b>0.04</b><br>(±0.00) | <b>0.82</b><br>(±0.44) | 2.50<br>(±0.00)        | <b>0.04</b><br>(±0.00) | <b>0.63</b><br>(±0.00) | <b>0.40</b><br>(±0.15) | <b>0.08</b><br>(±0.04) | 1.79<br>(±0.67)        | <b>0.68</b> |
| Negative Control           | 6.67<br>(±2.31)        | 5.33<br>(±1.79)        | 5.00<br>(±2.00)        | 4.80<br>(±1.79)        | 4.00<br>(±0.00)        | 5.00<br>(±2.00)        | 5.00<br>(±0.00)        | 4.00<br>(±0.00)        | 3.67<br>(±0.82)        | 3.60<br>(±0.89)        | 3.20<br>(±1.10)        | 4.00<br>(±0.00)        | 2.67<br>(±1.03)        | 5.33<br>(±2.31)        | 2.00<br>(±0.00)        | 2.00<br>(±0.00)        | 3.33<br>(±1.03)        | 3.60<br>(±0.89)        | 4.07        |
| Culture Control            | >8.00<br>(±0.00)       | >8.00<br>(±0.00)       | >8.00<br>(±0.00)       | >8.00<br>(±0.00)       | >8.00<br>(±0.00)       | >8.00<br>(±0.00)       | >8.00<br>(±0.00)       | >8.00<br>(±0.00)       | >8.00<br>(±0.00)       | >8.00<br>(±0.00)       | >8.00<br>(±0.00)       | >8.00<br>(±0.00)       | >8.00<br>(±0.00)       | >8.00<br>(±0.00)       | >8.00<br>(±0.00)       | 8.00<br>(±0.00)        | >8.00<br>(±0.00)       | >8.00<br>(±0.00)       | >8.00       |

\*bold indicates noteworthy minimum inhibitory activity.

All 23 EOs had a mean MIC value of 1.00 mg/mL, except for *A. sativum* (0.13 mg/mL) with the lowest MIC value, against a resistant clinical strain of *P. aeruginosa* (Resistant Clinical 2). This strain of *P. aeruginosa* (Resistant Clinical 2) was found to be resistant to the majority of the antibiotics tested in the antimicrobial susceptibility studies. *Vetiveria zizanioides* had a MIC value of 0.13 mg/mL against a clinical (Susceptible Clinical strain) and an ATCC-resistant (ATCC 33592) strain of *S. aureus*. *Santalum austrocaledonicum* had the second lowest MIC value recorded (0.19 mg/mL) against the clinical strain of *E. faecium*. The positive control, ciprofloxacin (0.01 mg/mL), acted as an effective broad-spectrum antibiotic against the ESKAPE pathogens. The negative control, water diluted in acetone (32.00 mg/mL), exhibited negligible bacterial inhibition against all 18 strains of bacteria.

The broth microdilution assay (Table 2) was used to screen the 23 EOs and select five to undergo further investigation.

Based on the minimum inhibitory activity, the five EOs comprise two oils (*O. vulgare* and *T. vulgaris*) that exhibit overall noteworthy activity ( $\leq 1.00$  mg/mL), one (*C. carvi*) that exhibits moderate overall antimicrobial activity (1.00 - 3.00 mg/mL), and two oils (*C. myrrha* and *M. recutita*) that on average exhibit poor antimicrobial activity ( $\geq 3.00$  mg/mL).

The reason EOs with varying inhibitory activity were chosen was to establish if there is a varied relationship between minimum inhibitory activity and resistance mechanisms. The average MIC of *O. vulgare*, *T. vulgaris*, *C. carvi*, *C. myrrha* and *M. recutita* were 0.78, 0.92, 1.62, 3.04 and 3.36 mg/mL respectively.

There were considerable variations in the MICs reported on the EOs found in this study when compared to the literature. This was likely due to the observed differences in the chemical composition which largely affects the overall MIC (Burt 2004). However, there were similarities

found in the inhibitory activity of *S. austrocaledonicum* and *V. zizanioides* in recent studies done by Orchard et al. (2017) and Singh (2022), despite differences in their composition. Thus, emphasizing there are many factors that could affect minimum inhibitory activity, not limited to the chemical composition of EOs.

For EOs to be considered as a viable solution to combat AMR, it needs to be effective against current multi-drug pathogens. Meropenem is a last-line antibiotic reserved for therapeutic use against serious infections that are resistant to the primary choice of antibiotic (CDC, 2019). The clinical strains of *A. baumannii* and *E. faecium* were identified as resistant to the meropenem and were further classified as multi-drug resistant. However, three ( $\pm$  13.00%) EOs displayed noteworthy inhibition against *E. faecium*, while 10 ( $\pm$  43.00%) EOs demonstrated noteworthy inhibition of *A. baumannii*. This strengthens the evidence and theoretical implications of EOs as potential solutions to combat AMR.

The minimum inhibitory activity of the selected EOs has been extensively studied in scientific papers and has reported noteworthy activity against one or more of the ESKAPE pathogens (Singh et al. 2015, Sakkas et al. 2016, Orchard et al. 2017, Bučková et al. 2018, Khalaf and Zahra 2020, Frydrysiak et al. 2021, Benameur et al. 2022). In this study EOs were most effective against *S. aureus*, followed by *P. aeruginosa* and *K. pneumoniae*, respectively. Essential oils had the lowest inhibitory effect on both strains of *E. faecium* and the reference strain of *A. baumannii* ATCC 19606. The EOs showed the most noteworthy activity against the resistant and susceptible clinical strains of the ESKAPE pathogens compared to the reference strains. This trend was also observed in previous studies by Sienkiewicz et al. (2012) and Singh (2022), where the MIC values of the resistant and clinical strains were lower than the reference strains, however, in some pathogens they were comparable. The variability in susceptibility between different strains

of the same pathogen accentuates the importance of exploring the inhibitory activity of EOs against a range of strains, incorporating reference, resistant, and clinical variants. The scrutiny of clinical strains provides a more realistic and informed perspective on the inhibitory characteristics of EOs. While reference strains lack prior exposure to antimicrobial agents, clinical strains are expected to have encountered such agents previously; it is plausible that the mechanism through which pathogens acquire resistance to antibiotics renders them more susceptible to inhibition by EOs.

### ***Anti-quorum sensing***

The anti-QS activity of the five (*Thymus vulgaris*, *O. vulgare*, *C. carvi*, *C. myrrha*, and *M. recutita*) selected EOs was investigated to consider their ability to prevent signalling responses. Percentage inhibition of  $\geq 70.00\%$  is indicative of noteworthy anti-QS activity and can be explained by the phenomenon of herd immunity (Jansen et al. 2021). Table 3 represents the percentage inhibition of violacein production (anti-QS activity), the MQSIC, and the MQSBC for the five selected EOs. All five EOs exhibited notable anti-QS activity, with a percentage inhibition ranging from 73.33 to 93.08%, when tested at a concentration of  $\leq 1.00$  mg/mL. The MQSBC was conducted to determine whether the observed percentage inhibition resulted from a reduction in violacein production (decreased QS signalling) rather than the death of the micro-organism. The average MQSBC values for all five EOs have been documented in Table 3.

*Thymus vulgaris* exhibited the greatest degree of inhibition (93.08%), signifying the highest anti-QS activity, at a concentration of 0.13 mg/mL, when compared to the other EOs that were examined. This particular EO had the best ability to inhibit QS which is supported by the percentage inhibition being closest to 100% (complete inhibition) at a low concentration.

*Origanum vulgare* exhibited anti-QS activity at an even lower concentration range than *T. vulgaris*, however, the greatest percentage inhibition of violacein was 85.63%.

Table 3. Anti-quorum sensing activity of the five selected essential oils.

| Samples                    | MQSIC (mg/mL) * <sup>1</sup> | Percentage inhibition (%) | MQSBC * <sup>2</sup> (mg/mL (± SD)) |
|----------------------------|------------------------------|---------------------------|-------------------------------------|
| <i>Carum carvi</i>         | 0.03 - 0.06                  | 74.82 - 82.50             | 2.69 (± 1.86)                       |
| <i>Commiphora myrrha</i>   | 0.50 - 1.00                  | 74.47 - 75.04             | 4.00 (± 0.00)                       |
| <i>Matricaria recutita</i> | 1.00                         | 73.33 - 92.73             | > 4.00 (± 0.00)                     |
| <i>Origanum vulgare</i>    | 0.03 - 0.06                  | 77.58 - 85.63             | 0.25 (± 0.00)                       |
| <i>Thymus vulgaris</i>     | 0.02 - 0.50                  | 70.14 - 93.08             | 0.42 (± 0.12)                       |

\*<sup>1</sup>MQSIC = Minimum quorum sensing inhibitory concentration. \*<sup>2</sup>MQSBC = Minimum quorum sensing bactericidal concentration. Positive control in water (0.31 mg/mL) 76.45 - 80.93 %, Positive control in acetone (0.01 - 0.08 mg/mL) 70.14 - 92.23 % and Culture control (> 8.00 mg/mL) growth at all concentrations.

*Carum carvi* and *O. vulgare* displayed similar anti-QS activity, MQSIC values and percentage inhibition, however, the MQSBC of *C. carvi* was considerably higher than the MQSBC of *O. vulgare*. It was also observed that the bactericidal activity of *C. carvi* was the most inconsistent (SD ± 1.86). Although *M. recutita* has the second highest percentage inhibition (92.73%), it was at a higher MQSIC value in comparison to *C. carvi*, *O. vulgare* and *T. vulgaris*. Ideally, the EOs should have a low MQSIC value with a high percentage inhibition (Mahumane 2016a). The MQSIC serves as an indicator of the EO's potency, with oils that exhibit significant percentage inhibition (≥ 70.00%) at lower MQSIC values considered more potent. For instance, in this study, both *T. vulgaris* and *M. recutita* demonstrated notable anti-QS activity at comparable percentages with only a minor difference of 0.35%. However, *T. vulgaris* displayed a lower MQSIC range (0.02 to 0.50 mg/mL) compared to *M. recutita*, which only exhibited substantial inhibition at 1.00 mg/mL. This indicates that *T. vulgaris* was the more potent oil in terms of anti-QS activity. Moreover, *T. vulgaris* had a lower MQSBC throughout the study underscoring that this oil not only disrupts bacterial communication but also effectively eliminates bacterial growth.

The current body of research on the anti-QS activity of the five EOs selected in this study is limited (Vattem et al. 2007, Al-Haidari et al. 2016, Alibi et al. 2020, Ghannay et al. 2022). The anti-QS activities of the five EOs exhibit a concentration-dependent relationship. However, this relationship is not consistently linear, which aligns with the findings from Mahumane (2016) and Poli et al. (2018). Additionally, this study found that *T. vulgaris*, *O. vulgare*, *C. carvi*, and *M. recutita* potentiated QS activity at concentrations below the MQSIC values. This finding was supported by other studies investigating anti-QS of other EOs (Vattem et al. 2007, Al-Haidari et al. 2016, Mahumane 2016, Alibi et al. 2020, Ghannay et al. 2022). The findings from this study, along with recent research, emphasizes that QS is highly dependent on concentration. Quorum sensing must be meticulously regulated when used as an antimicrobial agent due to its narrow effectiveness range as a QS inhibitor. It's crucial to note that concentrations below the MQSIC can potentiate QS activity. Unlike microbial inhibition, the inhibitory effect of QS does not follow a linear pattern. Al-Haidari et al. (2016) reported that *C. carvi* did not show any anti-QS activity, however, the disc diffusion method was used, which does not accommodate the volatile and lipophilic nature of EOs, highlighting that varying methods can lead to different conclusions in the results. *Carum carvi* exhibited a QS inhibition of 47.57% at a concentration of 10.00 mg/mL in a recent study (Ghannay et al. 2022). However, these findings contradict the results of the present study, which may be due to discrepancies in the methodology where Ghannay et al. (2022) utilized a microdilution technique and a different approach to quantifying the inhibition of violacein.

*Origanum vulgare* and *T. vulgaris* both inhibited violacein production in a study conducted by Vattem et al. (2007), with *O. vulgare* exhibiting a percentage inhibition of 75%. This research also revealed that *T. vulgaris* played a role in mediating violacein production, aligning with the findings of this study. Alvarez et al. (2014) identified *O. vulgare* as a powerful QS inhibitor. This

finding was supported by Čobanović et al. (2023), which reported anti-QS activity from *O. vulgare* and *T. vulgaris* at concentrations comparable to those observed in our research.

A study reported notable anti-QS activity (66.00% - 100.00%) by *C. myrrha* at a concentration of 1.70 mg/mL when using *C. violaceum*, which is comparable to the findings in this study (Rubegeta et al. 2019). Křížková et al. (2023) used *Vibrio campbellii* as the biosensor strain when investigating the anti-QS properties of *M. recutita*. Their results highlighted the potent inhibitory effects demonstrated by this EO, even at remarkably low concentrations. However, it is important to acknowledge that the methodologies employed to assess the level of inhibition differed from those utilized in the present study, as theirs focused on the response of pathogens to autoinducers in the presence of bioluminescent signals. The body of research on the anti-QS activity of *C. carvi* and *M. recutita* is quite limited compared to the other three EOs.

### ***Biofilm inhibition***

Figure 1 demonstrates the average noteworthy biofilm inhibition ( $\geq 70.00\%$ ) at different concentrations of each of the five EOs investigated. *Origanum vulgare* and *T. vulgaris* exhibited noteworthy biofilm inhibition ( $\geq 70.00\%$ ) at four different concentrations including 0.05 mg/mL, the lowest concentration that inhibited biofilm formation (Figure 1). *Carum carvi*, *M. recutita*, and *C. myrrha* inhibited  $\geq 70.00\%$  of biofilm formation between 2.00 - 4.00 mg/mL. These three oils also potentiated biofilm formation between concentrations of 0.13 mg/mL and 1.00 mg/mL, which is denoted by a negative percentage (data available on request). In the current study, noteworthy biofilm inhibition was reported at 0.31  $\mu\text{g/mL}$  for ciprofloxacin, 0.31  $\mu\text{g/mL}$  for erythromycin, and 1.25  $\mu\text{g/mL}$  for gentamicin. The culture control produced the desired OD between 0.77 and 1.25, concurring that *S. aureus* ATCC 6538 is a strong biofilm-forming strain (Abbas et al. 2019). The

negative control, water in acetone made to a concentration of 32.00 mg/mL, did not notably inhibit biofilm formation at any of the concentrations tested (0.13 - 4.00 mg/mL).

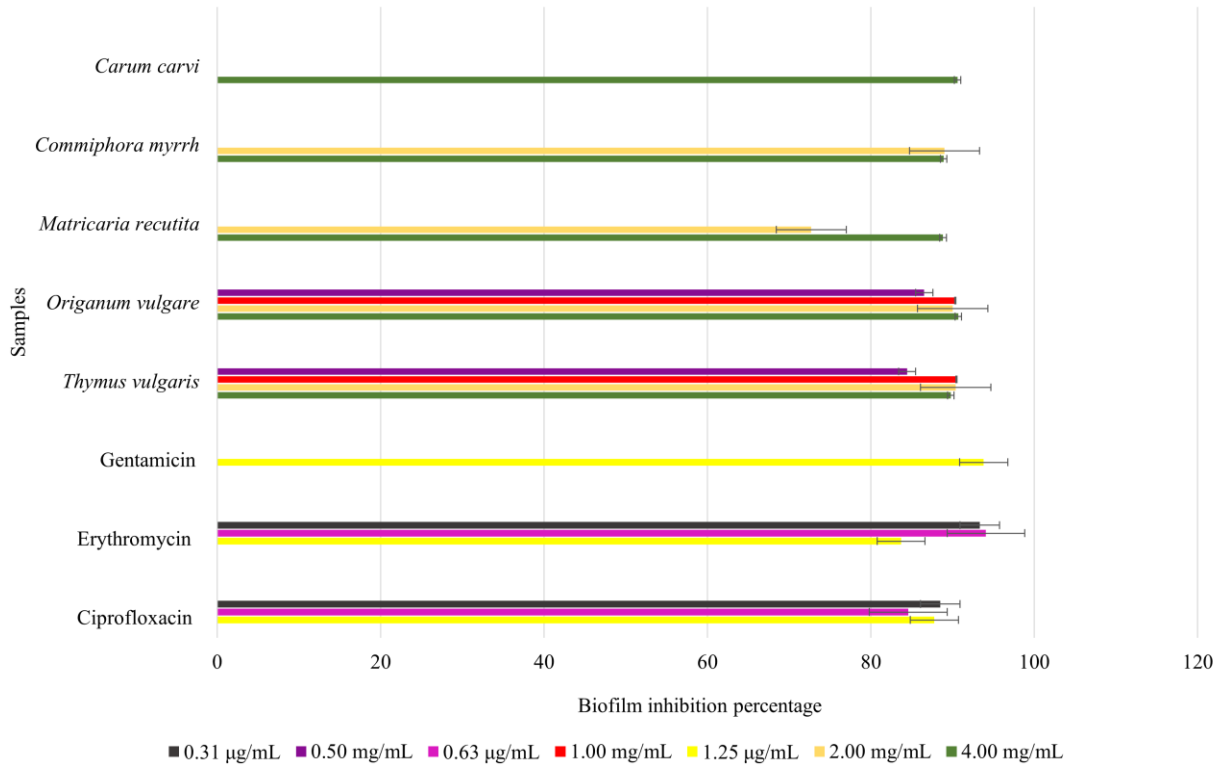


Figure 1. The biofilm inhibition of five EOs on the *S. aureus* ATCC 6538 strain (expressed as an average percentage inhibition).

Jafri et al. (2014) found that at all concentrations (0.20 - 12.80% v/v) of *T. vulgaris* inhibited more than 70.00% of biofilm formation against clinical strains of *S. aureus*. A study assessing the biofilm inhibitory activity and antimicrobial activity of *O. vulgare* against three different strains of *S. aureus* (methicillin-sensitive *S. aureus*, methicillin-resistant *S. aureus* and *S. aureus* ATCC 29213) concluded that at 0.06 mg/mL,  $\geq 93.07\% \pm 0.37$  biofilm formation was inhibited (Ersanli et al. 2023). These results exhibit increased biofilm inhibition activity at lower concentrations in comparison to the findings in this study, likely due to *S. aureus* ATCC 6538 having greater biofilm-

forming properties (Koçak et al. 2022). Stojanović-Radić et al. (2021) also corroborated that *O. vulgare* and *T. vulgaris* were both strong biofilm inhibitors against clinical strains of *S. aureus*, inhibiting up to 96.00% of biofilm formation.

Liu et al. (2023) reported that *C. carvi* inhibited the formation of MRSA biofilm completely at a concentration of 1.28% (v/v), however, the minimum bactericidal concentration of this strain was 0.64%, thus if cidal activity occurred, biofilm inhibition would have not been possible. A study in 2014, investigated the anti-biofilm, anti-hemolysis, and anti-virulence activities of 83 EOs, which included the five EOs selected in the current study (Lee et al. 2014). Lee et al. (2014) found that *C. myrrha* (89.0% biofilm inhibition), *T. vulgaris* (78.0% biofilm inhibition), and *O. vulgare* (77.0% biofilm inhibition), demonstrated notable biofilm inhibitory activity against *S. aureus* ATCC 6538. While *C. carvi* and *M. recutita* did not show biofilm inhibition and were found to be more unstable than the previously three mentioned EOs (Lee et al. 2014). Although the chemistry of *C. myrrha* and *M. recutita*, could have differed from this study, it does highlight the inconsistency across chemotypes, rendering them impractical alternatives due to their erratic activity. In addition, to variations in the chemotypes, careful consideration should be exercised when comparing biofilm inhibition activity as there are several methods of washing techniques used in the biofilm assay which could lead to discrepancies in the outcomes (O'Toole 2011, Xu et al. 2016, Shukla and Toleti 2017, Ersanli et al. 2023).

### ***Resistance induction***

A total of 20 passages were completed to assess the effect of the five EOs on the induction of resistance of *S. aureus* ATCC 6538. The MIC values, over the 20 passages, were plotted graphically, along with the MIC values of the three conventional antibiotics tested (Figure 2 - 6).

*Carum carvi* is representative of an EO with moderate antimicrobial activity. The results calculated for all 20 passages (shown in Figure 2), revealed that its inhibitory activity was inconsistent, displaying significant variance ( $SD \pm 1.24$ ,  $p < 0.05$ ) in the MIC value. At passages 15 and 16, *C. carvi* exhibited noteworthy inhibition (1.00 - 1.50 mg/mL), thereafter it returned to its original MIC value of 2.00 mg/mL (moderate antimicrobial activity). There was a sudden increase in the EOs MIC value at passage 19 (6.00 mg/mL) before reverting to the typical range observed during the other passages (2.00 - 4.00 mg/mL). Although significant variance ( $SD \pm 1.24$ ,  $p < 0.05$ ) across the entire study was observed, the significant variations were limited to individual passages such as passages 7-9 ( $p = 0.03$ ), indicating that there was no permanent change in the MIC value and thus the absence of any development of long-term resistance. The fluctuations observed in the MIC values between passages 14 and 17 were deemed statistically insignificant ( $p$ -value = 0.43). In addition, the fluctuations between passages 18 and 20 were also not statistically significant ( $p$ -value = 0.15). This suggests that although no persistent resistance was observed, there was a notable yet temporary alteration in the MIC value.

The sub-inhibitory exposure of *C. carvi* led to a minor shift in the antibiotic MICs ( $SD \pm 0.29$ ). Ciprofloxacin exhibited a sudden increase in the MIC value (from 0.20 to 1.25  $\mu$ g/mL) at passage 17, and then dropped thereafter. The antimicrobial activity of erythromycin over the 20 passages had the least variance ( $SD \pm 0.20$ ) of the three antibiotics tested. There was a sudden dip in the MIC of gentamicin at passages 6, 9 and 18, these were denoted as noteworthy as they were more than two serial dilutions, however, the overall MIC value was consistent ( $SD \pm 0.39$ ).

*Commiphora myrrha*, although classified as an oil with poor antimicrobial efficacy against the ESKAPE pathogens, displayed MIC values ranging between 0.25 - 4.00 mg/mL ( $SD \pm 1.18$ ) (Figure 3). An intriguing finding was that *C. myrrha* exhibited an increase in its antimicrobial

activity, from 4.00 mg/mL at passage 13 to 0.05 - 0.25 mg/mL between passages 14-16. This was followed by a reverse trend at passage 17, where the MIC value increased by four serial dilutions from 0.25 to 4.00 mg/mL. These two fluctuations are considered noteworthy as the change was more than two serial dilutions. This was further supported by statistical assessment indicating that the changes in the MIC value from passages 16 to 20 were significant ( $p$ -value = 0.03); however, by passage 20, *C. myrrha* reverted to the standard range observed in preceding passages. The overall trend of *C. myrrha*'s antimicrobial activity predominantly aligned with its poor efficacy, and there were no consistent alterations observed in the bacterial strain due to repeated exposure to the EO.

For the strain exposed to *C. myrrha* at sub-inhibitory concentrations, small variations were observed for the MICs of the antibiotics. Ciprofloxacin exhibited no sudden significant increases or decreases in the MIC value between consecutive passages, and the MIC value ranged between 0.31 to 1.25  $\mu$ g/mL (1.25  $\mu$ g/mL was only reported at passage 7). Erythromycin was the most consistent antimicrobial (between *C. myrrha* and the antibiotics) over the 20 passages (SD  $\pm$  0.17), the MIC value ranged between 0.16 and 0.63  $\mu$ g/mL. Gentamicin had a sudden increase in the MIC value at passage 7 from 0.16 to 1.25  $\mu$ g/mL. At passage 17, another spike in the MIC value was observed in gentamicin, spiking to 0.94  $\mu$ g/mL before decreasing to 0.08  $\mu$ g/mL at passage 18.

*Matricaria recutita* EO demonstrated a MIC value between 2.00 - 4.00 mg/mL throughout the majority of the passages, with the exception of passage 16 where the MIC value was 0.75 mg/mL (SD  $\pm$  0.97) (Figure 4). The changes between passages 14 and 17, were determined to be not significant ( $p$ -value = 0.79). The minimum inhibitory activity of the antibiotics over the 20 passages of sub-inhibitory exposure of *M. recutita* was more consistent. The MIC value of

ciprofloxacin (SD  $\pm$  0.27) and erythromycin (SD  $\pm$  0.19) remained stable against *S. aureus* over the 20 passages. A minor increase observed in the MIC value of ciprofloxacin at passage 7 (1.25  $\mu$ g/mL), was not statistically significant ( $p > 0.05$ ). There were sudden significant ( $p < 0.05$ ) increases observed in the MIC value of gentamicin after exposure to *M. recutita* at passage 7 (from 0.16 to 0.94  $\mu$ g/mL). This observation suggests that exposure to *M. recutita* resulted in a transient decrease in the susceptibility of *S. aureus* to gentamicin.

*Origanum vulgare* had the least variation in the MIC values (SD  $\pm$  0.21) over the 20 passages in comparison to all five EOs (Figure 5). The MIC values ranged between 0.50 -1.00 mg/mL against *S. aureus* ATCC 6538. The average SD of the antibiotics (SD  $\pm$  0.45) was higher than that of the EO, *O. vulgare* (SD  $\pm$  0.21). The MIC values of all three antibiotics (ciprofloxacin, erythromycin and gentamicin) decreased between passages 7 and 8 from 2.50  $\mu$ g/mL, 2.50  $\mu$ g/mL and 1.25  $\mu$ g/mL to 0.31  $\mu$ g/mL, 0.31  $\mu$ g/mL and 0.23  $\mu$ g/mL respectively. This was a decrease of more than two serial dilutions and is highlighted as a noteworthy fluctuation in the induction of the resistance pattern. Additionally, an increase in the MIC values of erythromycin from 0.31  $\mu$ g/mL at passage 6 to 2.50  $\mu$ g/mL at passage 7 was observed. Gentamicin exhibited a noteworthy increase of two serial dilutions, between passages 17 and 18 (from 0.08 - 0.47  $\mu$ g/mL).

*Thymus vulgaris* had the second least variation in MIC (SD  $\pm$  0.32) over the 20 passages (Figure 6). The MIC values of *T. vulgaris* ranged between 0.50 -1.00 mg/mL for the majority of the passages, with the exception of passage 18, where a MIC value of 2.00 mg/mL was reported, however, it was not statistically significant ( $p > 0.05$ ). The MIC values of all three antibiotics spiked at passage 8 (2.50  $\mu$ g/mL) before returning to 0.31  $\mu$ g/mL for ciprofloxacin and erythromycin, and 0.23  $\mu$ g/mL for gentamicin. At passage 19, the MIC of gentamicin increased from 0.16  $\mu$ g/mL to 1.25  $\mu$ g/mL, which is also a notable increase in the MIC value.

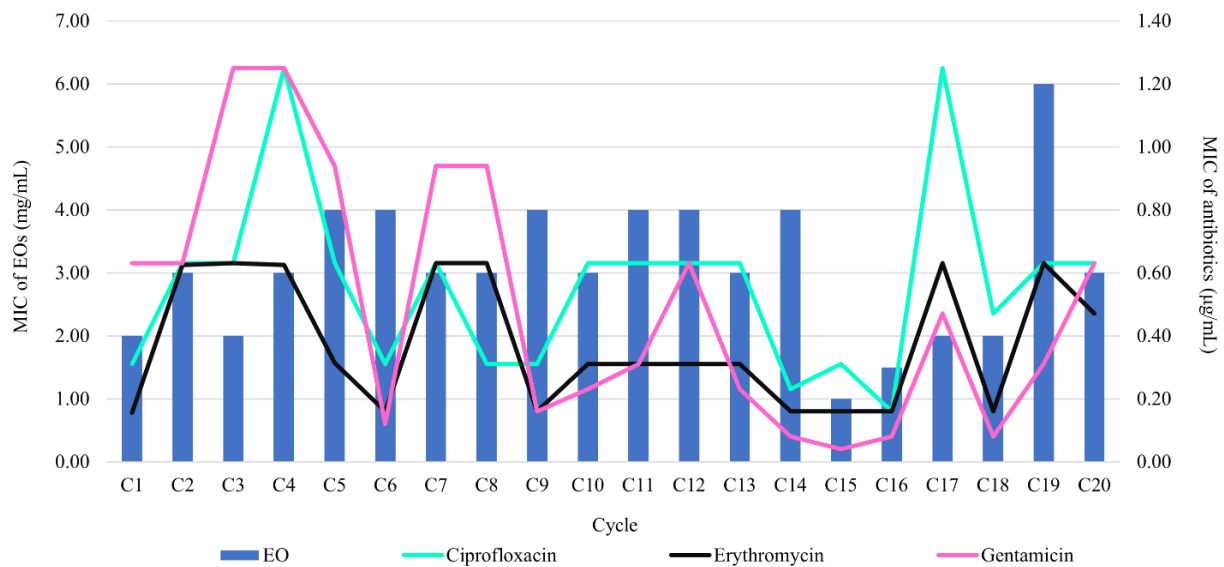


Figure 2. The minimum inhibitory concentration of *C. carvi* and three conventional antibiotics, ciprofloxacin, erythromycin, and gentamicin, over 20 passages against *S. aureus* ATCC 6538.

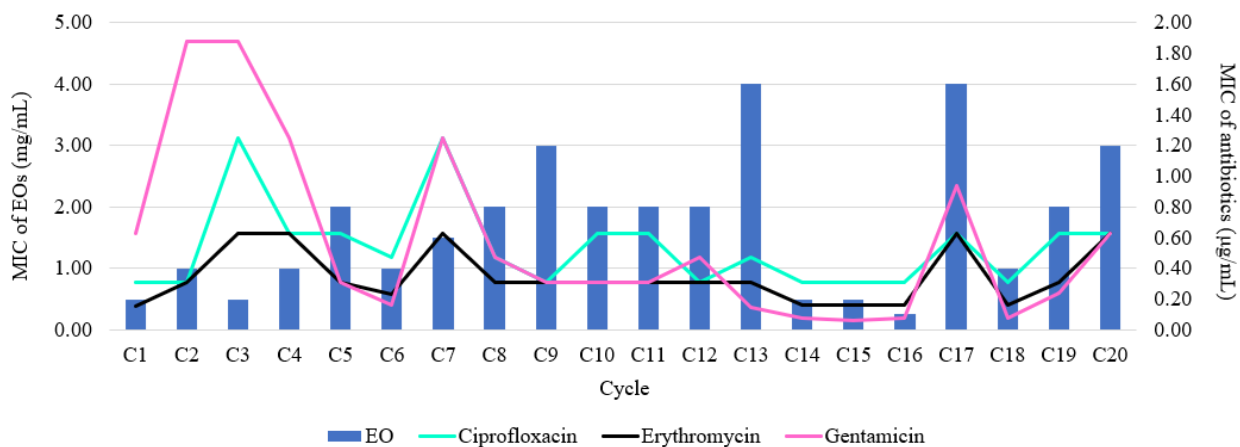


Figure 3. The minimum inhibitory concentration of *C. myrrha* and three conventional antibiotics, ciprofloxacin, erythromycin, and gentamicin, over 20 passages against *S. aureus* ATCC 6538.

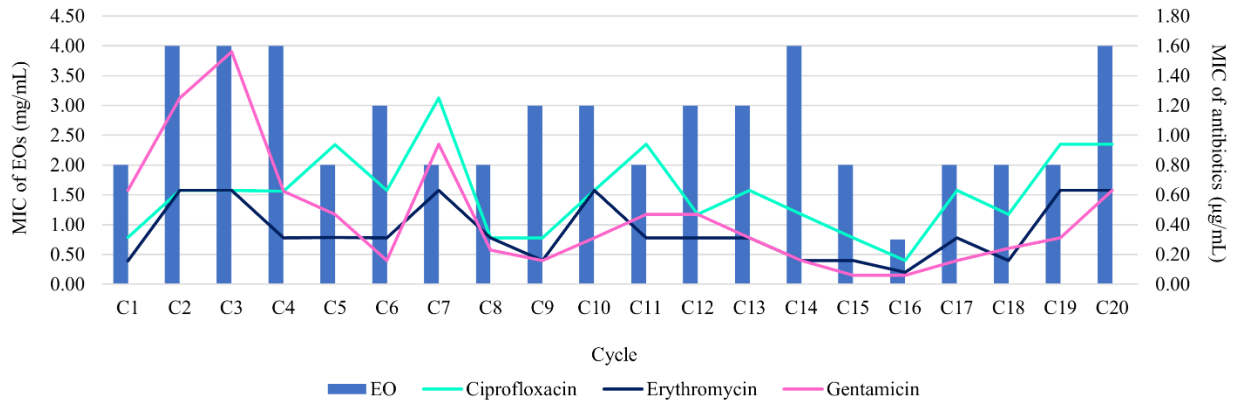


Figure 4. The minimum inhibitory concentration of *M. recutita* and three commonly used antibiotics, namely ciprofloxacin, erythromycin, and gentamicin, throughout 20 passages against *S. aureus* ATCC 6538.

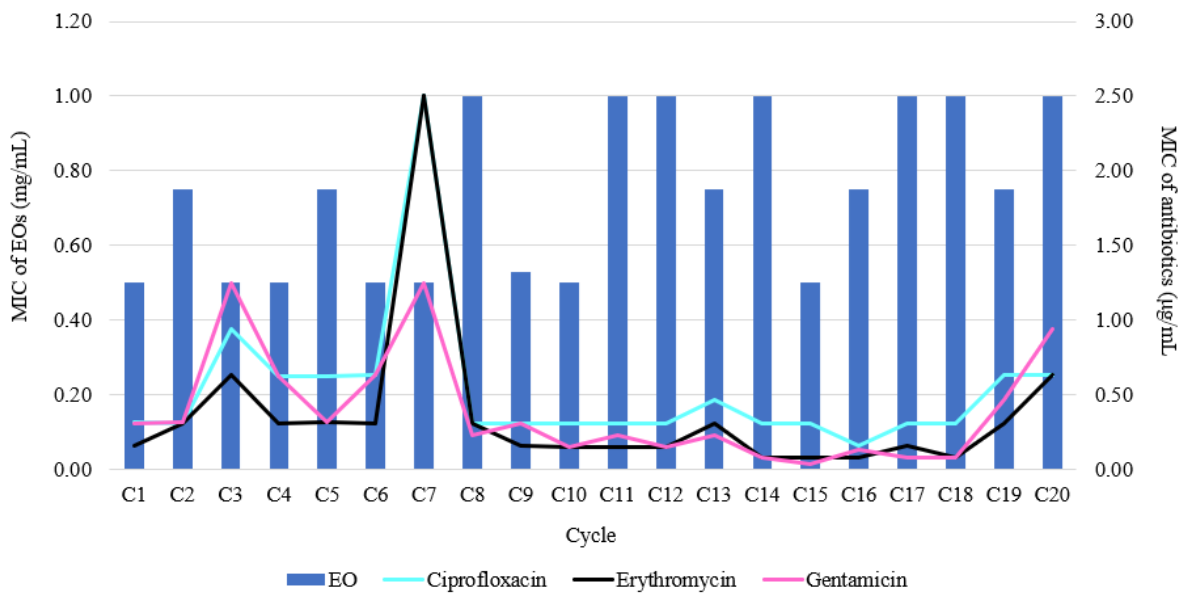


Figure 5: Minimum inhibitory concentration profiles of *O. vulgare* and three conventional antibiotics (Ciprofloxacin, Erythromycin, Gentamicin) across 20 passages against *S. aureus* ATCC 6538.

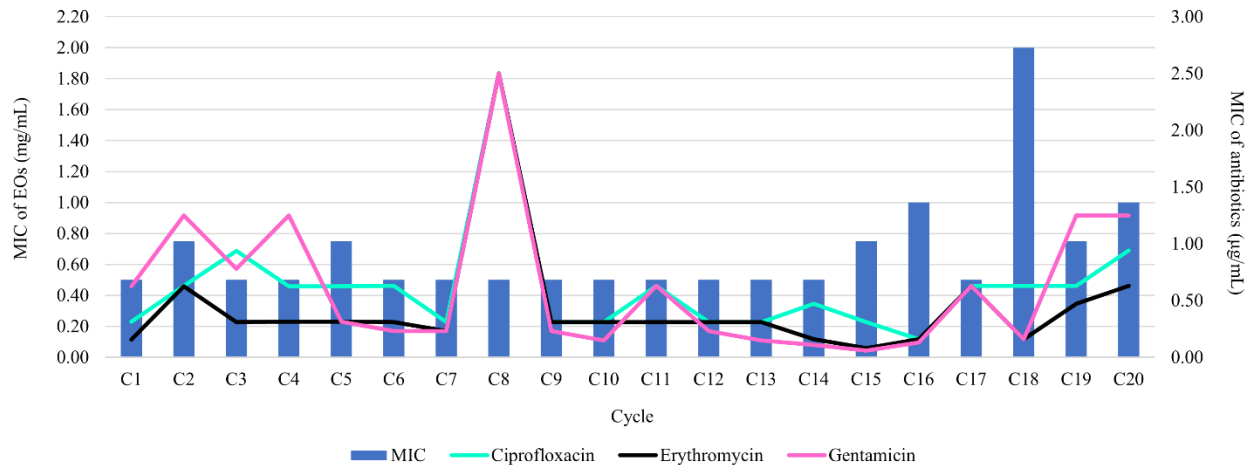


Figure 6. The minimum inhibitory concentration of *T. vulgaris* and three conventional antibiotics, ciprofloxacin, erythromycin, and gentamicin, was over 20 passages against *S. aureus* ATCC 6538.

Overall, no permanent resistance was detected by *S. aureus* ATCC 6538 due to the repeated exposure to the EOs. The fluctuations in the MIC values of the five EOs were transient in nature. A study conducted by Speck et al. (2020), which investigated the induction of resistance in *S. aureus* after exposure to the antimicrobial agent sodium hypochlorite, found that fluctuations were also temporary. This suggests occurrences of heterogeneity of the pathogen, *S. aureus* ATCC 6538, caused by repeated exposure to an antimicrobial agent. A study that is most similar to the methodology employed in this study for the induction of resistance assay was performed by Hammer et al. (2012), focusing on *M. alternifolia* and one of its major components, terpinen-4-ol. It was previously reported that changes in the MIC value of *M. alternifolia* against *S. aureus* reference strain ATCC 25923, when exposed over a six-day period, were evident yet not significant, as they did not increase by more than one doubling dilution which was the threshold set by this study (Hammer et al. 2012).

Typically, resistance is understood to be an inevitable characteristic that arises from either the acquisition of resistance genes through horizontal gene transfer or mutations in natural genes

(Munita and Arias 2016). However, it is important to note that resistance can also be temporary and manifest only in subpopulations, leading to phenotypic variation within the bacterial culture (Andersson et al. 2019). This phenomenon, known as heteroresistance, was first described in the 1940s and involves the presence of subpopulations of cells within a bacterial isolate that demonstrate heightened resistance or decreased susceptibility to antibiotics compared to the main population (Alexander and Leidy 1947). The data in this study demonstrates patterns of heteroresistance against conventional antibiotics, due to repeated exposure to sub-inhibitory concentrations of the EOs. Numerous studies, both *in vitro* and *in vivo*, have indicated that the survival of these small subpopulations of resistant bacteria can occur in infected hosts, contributing to a heightened emergence of resistance (Tello et al. 2012, Andersson et al. 2019, Pereira et al. 2021). Heteroresistance is a common occurrence among various bacterial species and antibiotics, and it is often characterized by its inherent instability. This instability manifests as an intermittent or temporary expression of resistance, typically in response to stressors like exposure to antimicrobials, such as an EO, although not consistently occurring on every occasion. In certain instances, the subpopulations displaying resistance can revert to susceptibility after a limited number of generations of growth in the absence of antimicrobial pressure (Tello et al. 2012). This was also observed in our study, where *S. aureus*, after being exposed to *C. carvi* for the 19th time and subsequently removed, reverted to its original MIC value for gentamicin. None of the changes in the MIC of the antibiotics were permanent, which is a characteristic of heteroresistance. Heteroresistance arose due to exposure to sub-inhibitory concentrations of the EOs, which highlights a need for caution around the use of essential oils at sub-inhibitory concentrations.

The susceptibility of *S. aureus* to the antibiotics penicillin, chloramphenicol, kanamycin, vancomycin, and erythromycin was assessed by examining the impact of adaptation to a sub-

inhibitory concentration of eugenol and citral (EO compounds) (Apolónio et al. 2014). It was found that resistance to the conventional antibiotics was not induced due to exposure to the EO compounds. This proposes that the compounds of *O. vulgare* (carvacrol, *p*-cymene, *c*-terpinene) and *T. vulgaris* (thymol,  $\gamma$ -terpinene, *p*-cymene) should be investigated further, to see if their compounds could maintain the noteworthy activity whilst reducing the incidence of heteroresistance.

It was observed, in this study, that there was a substantial change in the growth kinetics of *S. aureus* ATCC 6538, specifically the exponential phase of the bacteria shifted from 3 hrs to 11 hrs between passages 16 and 17 (data available on request). However, after an additional passage, the exponential phase reverted. It can be deduced that these changes are a result of repeated exposure to the EOs. A study by Vázquez-Sánchez et al. (2015) found that bacteria exposed to sub-lethal concentrations of *T. vulgaris* EO triggered cellular adaptations. These adaptations rendered the oil ineffective in completely eradicating the biofilm. Furthermore, two additional studies corroborated that changes in the duration of the exponential phase could support bacterial survival and may promote regrowth once the antimicrobial is removed (Rolfe et al. 2012, Li et al. 2016). Therefore, conducting further research on how essential oils impact bacterial growth during specific phases would be beneficial in gaining a deeper understanding of their effects on resistance.

### ***Overview of all four assays***

This study illuminated the relationships between EOs with varying inhibitory activity against planktonic bacteria and resistance mechanisms as well as induction of resistance capability. The noteworthy activity of the five EOs for all four assays is tabulated to highlight the relationship observed (Table 4). A trend was observed between the EOs classified by their minimum inhibitory

activity as noteworthy, moderate, or poor. *Thymus vulgaris* and *O. vulgare* were two EOs with a noteworthy inhibitory activity that additionally demonstrated notably broad-spectrum activity in all four assays. Furthermore, these two oils were able to sustain their MIC value with little deviation over the 20 passages in comparison to *M. recutita*, *C. carvi* and *C. myrrha*, with the latter two showing greater variability. Essential oils with moderate and poor inhibitory activity had notable anti-QS and biofilm inhibition activity, however, at higher concentrations and/or at a lower percentage of inhibition. After each passage of sub-inhibitory exposure of *S. aureus* to the EOs, it was found that *M. recutita*, *C. carvi* and *C. myrrha* exhibited relatively fewer occurrences of heteroresistance. Resulting in a lower impact on the antibiotics' ability to sustain their inhibitory activity compared to *T. vulgaris* and *O. vulgare*.

## **Conclusion**

This study investigated the potential use of EOs as antimicrobials to combat AMR, especially against current multi-drug resistant pathogens. When examining the inhibitory activities, two EOs (*O. vulgare* and *T. vulgaris*) demonstrated noteworthy and broad-spectrum activity against multi-drug resistant bacterial strains.

The five EOs studied were able to inhibit resistant mechanisms such as QS and biofilm formation. The highest percentage inhibiting QS was 93.08% by *T. vulgaris*. Furthermore, when biofilm studies were undertaken, it was found that EOs with noteworthy minimum inhibitory activity demonstrated greater biofilm inhibition than EOs with moderate and poor inhibitory activity. *Thymus vulgaris* and *O. vulgare* inhibited >80.00% biofilm formation at 0.05 - 4.00 mg/mL.

No significant resistance was detected over the 20 passages in the resistance induction assay, however, instances of heteroresistance were observed by *S. aureus* against conventional antibiotics due to exposure to the EOs. It was observed that *T. vulgaris* and *O. vulgare* maintained their inhibitory activity after each subsequent exposure for a total of 20 passages. Moreover, they were able to inhibit QS and biofilm formation at sub-inhibitory concentrations, which was not the case for the oils with an overall moderate or poor inhibitory activity. Based on the five EOs under investigation, it was concluded that the ability of the EO to sustain their minimum inhibitory activity was based on their overall inhibitory activity (average MIC value against the ESKAPE pathogens).

This study demonstrates that EOs present a promising solution to the antimicrobial crisis. As more pathogens become multi-drug resistant or pan-resistant to antibiotics, the EOs (*O. vulgare* and *T. vulgaris*) proved to be effective against the current multi-drug resistant strains tested. These findings underscore the potential of EOs studied herein, demonstrating their effectiveness in preventing the development of resistance mechanisms against themselves, thereby hindering the emergence of resistance. While EOs offer a plausible solution, it is imperative to continue investigating their potential contribution to resistance against conventional antibiotics to ensure a comprehensive understanding of their therapeutic implications. Ideally, EOs such as *T. vulgaris* and *O. vulgare* with noteworthy activity in all four assays performed should be investigated in future studies to supplement these findings. Further recommendations for research in this area should include gene sequencing after each passage. This will provide information on precisely what mutations are occurring due to exposure to the EO, this will allow great insight when undertaking *in vivo* studies.

Table 4. Overview of noteworthy activities of all five essential oils in all four assays.

| Essential oil                                    | Minimum inhibitory activity             |  | Anti-QS activity       |                            | Biofilm inhibition     |                            | Induction of resistance                 |   |   |  |  |  |
|--|---|--|------------------------|----------------------------|------------------------|----------------------------|---|---|---|--|--|--|
|  | Average MIC against the ESKAPE<br>mg/mL | Classification of activity<br>Noteworthy, moderate or poor | Concentration<br>mg/mL | Percentage inhibition<br>% | Concentration<br>mg/mL | Percentage inhibition<br>% | MIC range over the 20 passages<br>mg/mL | *SD of EOs over the 20 passages<br>± 0.00 | *SD of ciprofloxacin over the 20 passages<br>± 0.00 | *SD of erythromycin over the 20 passages<br>± 0.00 | *SD of gentamicin over the 20 passages<br>± 0.00 | Average *SD of all three antibiotics<br>± 0.00 |
| <i>Origanum vulgare</i><br>(Oregano)             | 0.78                                    | Noteworthy   | 0.03 - 0.06            | 77.58 - 85.63              | 0.50 - 4.00            | 86.54 - 90.71              | 0.50 - 1.00                             | 0.21                                      | 0.49  | 0.52   | 0.36   | 0.45   |
| <i>Thymus vulgaris</i><br>(Thyme)                | 0.92                                    | Noteworthy   | 0.02 - 0.50            | 70.14 - 93.08              | 0.50 - 4.00            | 84.45 - 90.48              | 0.50 - 2.00                             | 0.35                                      | 0.48  | 0.50   | 0.60   | 0.53   |
| <i>Carum carvi</i><br>(Caraway)                  | 1.62                                    | Moderate   | 0.03 - 0.06            | 74.82 - 82.50              | 4.00                   | 90.61                      | 1.50 - 4.00                             | 1.12                                      | 0.28  | 0.20   | 0.39   | 0.29   |
| <i>Commiphora myrrha</i><br>(Myrrh)              | 3.12                                    | Poor   | 0.50 - 1.00            | 74.47 - 75.04              | 2.00 - 4.00            | 88.92 - 89.02              | 0.25 - 4.00                             | 1.10                                      | 0.27  | 0.17   | 0.56   | 0.33   |
| <i>Matricaria recutita</i><br>(German chamomile) | 3.36                                    | Poor   | 1.00                   | 73.33 - 92.73              | 2.00 - 4.00            | 72.69 - 88.84              | 0.75 - 4.00                             | 0.92                                      | 0.27  | 0.19   | 0.39   | 0.28   |

\*SD = standard deviation.

## **Acknowledgements**

The authors would like to thank Mrs. Phumzile Moerane for her laboratory assistance. Dr. Teena Thomas from NHLS Infection Control and Microbiology Laboratory is thanked for the supply of clinical strains.

## **Funding statement**

The National Research Foundation of South Africa is thanked for financial support via the Thuthuka funding instrument grant number 129672.

## **Conflict of Interest**

No conflict of interest was declared.

## **Data Availability**

The data underlying this article will be shared on reasonable request to the corresponding author.

## **References**

Abbas, H. A., Elsherbini, A. M., and Shaldam, M. A. 2019. Glyceryl trinitrate blocks staphyloxanthin and biofilm formation in *Staphylococcus aureus*. *African Health Sciences* 19 (1):1376-1384. doi:10.4314/ahs.v19i1.10.

- Ahmed, S. K., Hussein, S., Qurbani, K., Ibrahim, R. H., Fareeq, A., Mahmood, K. A., and Mohamed, M. G. 2024. Antimicrobial resistance: Impacts, challenges, and future prospects. *Journal of Medicine, Surgery, and Public Health* 2 (2024):100081. doi:10.1016/j.gjmedi.2024.100081.
- Al-Haidari, R. A., Shaaban, M. I., Ibrahim, S. R., and Mohamed, G. A. 2016. Anti-quorum sensing activity of some medicinal plants. *African Journal of Traditional, Complementary and Alternative Medicines* 13 (5):67-71. doi:10.21010/ajtcam.v13i5.10.
- Alexander, H. E., and Leidy, G. 1947. Mode of action of streptomycin on type b *H. influenzae*: I. Origin of resistant organisms. *The Journal of Experimental Medicine* 85 (4):329. doi:10.1084/jem.85.4.329.
- Alibi, S., Ben Selma, W., Ramos-Vivas, J., Smach, M. A., Touati, R., Boukadida, J., Navas, J., and Ben Mansour, H. 2020. Anti-oxidant, antibacterial, anti-biofilm, and anti-quorum sensing activities of four essential oils against multidrug-resistant bacterial clinical isolates. *Current Research in Translational Medicine* 68 (2):59-66. doi:10.1016/j.retram.2020.01.001.
- 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. d. 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* 5:699. doi:10.3389/fmicb.2014.00699.
- Andersson, D. I., Nicoloff, H., and Hjort, K. 2019. Mechanisms and clinical relevance of bacterial heteroresistance. *Nature Reviews Microbiology* 17 (8):479-496. doi:10.1038/s41579-019-0218-1.
- Apolónio, J., Faleiro, M. L., Miguel, M. G., and Neto, L. 2014. No induction of antimicrobial resistance in *Staphylococcus aureus* and *Listeria monocytogenes* during continuous exposure to eugenol

- and citral. *Federation of European Microbiological Societies Microbiology Letters* 354 (2):92-101. doi:10.1111/1574-6968.12440.
- Bali, E. B., Erdönmez, D., Yavuz, M., and Koca, U. 2021. Inhibitory effects of aromaterapeutic rose essential oil against bacterial quorum sensing and biofilm formation. *Journal of the Turkish Microbiology Society* 41 (3):245-253. doi:10.5222/TMCD.2021.72792.
- Bazargani, M. M., and Rohloff, J. 2016. Antibiofilm activity of essential oils and plant extracts against *Staphylococcus aureus* and *Escherichia coli* biofilms. *Food control* 61:156-164. doi:10.1016/j.foodcont.2015.09.036.
- Becerril, R., Nerin, C., and Gomez-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. doi:10.1089/fpd.2011.1097.
- Benameur, Q., Gervasi, T., Pellizzeri, V., Pfluchtová, M., Gruřová, D., Cicero, N., and Meriem-Hind, B. 2022. Comparison of sensitivity to a commercial *Origanum vulgare* essential oil between extended-spectrum  $\beta$ -lactamases (ESBL-) and non-ESBL-producing *Enterobacteriaceae* isolates. *Natural Product Research* 36 (11):2830-2835. doi:10.1080/14786419.2021.1933969.
- Bermúdez-Capdevila, M., Cervantes-Huamán, B. R. H., Rodríguez-Jerez, J. J., and Ripolles-Avila, C. 2022. Repeated sub-inhibitory doses of cassia essential oil do not increase the tolerance pattern in *Listeria monocytogenes* cells. *Lebensmittel-Wissenschaft & Technologie* 165:113681. doi:10.1016/j.lwt.2022.113681.
- Brun, P., Bernabè, G., Filippini, R., and Piovan, A. 2019. *In vitro* antimicrobial activities of commercially available tea tree (*Melaleuca alternifolia*) essential oils. *Current microbiology* 76:108-116. doi:10.1007/s00284-018-1594-x.

- Bučková, M., Puškárová, A., Kalászová, V., Kisová, Z., and Pangallo, D. 2018. Essential oils against multidrug resistant Gram-negative bacteria. *Biologia* 73 (8):803-808. doi:10.2478/s11756-018-0090-x.
- Burt, S. 2004. Essential oils: their antibacterial properties and potential applications in foods-A review. *International Journal of Food Microbiology* 94 (3):223-53. doi:10.1016/j.ijfoodmicro.2004.03.022.
- Cáceres, M., Hidalgo, W., Stashenko, E., Torres, R., and Ortiz, C. 2020. Essential oils of aromatic plants with antibacterial, anti-biofilm and anti-quorum sensing activities against pathogenic bacteria. *Antibiotics* 9 (4):147. doi:10.3390/antibiotics9040147.
- Camele, I., Elshafie, H. S., De Feo, V., and Caputo, L. 2019. Anti-quorum sensing and antimicrobial effect of Mediterranean plant essential oils against phytopathogenic bacteria. *Frontiers in Microbiology* 10:2619. doi:10.3389/fmicb.2019.02619.
- Chakraborty, M., Bardhan, T., Basu, M., and Bhattacharjee, B. 2022. Influence of sub-inhibitory dosage of cefotaxime on multidrug resistant *Staphylococcus haemolyticus* isolated from sick neonatal care unit. *Antibiotics* 11 (3):360. doi:10.3390/antibiotics11030360.
- CLSI. 2020. Clinical and Laboratory Standards Institute Performance standards for antimicrobial susceptibility testing. *Clinical and Laboratory Standards Institute* 30th edition (CLSI supplement M100):32-332. ISBN:978-1-68440-220-5.
- Čobanović, R., Čabarkapa, I., Kokić, B., Rakita, S., and Tomičić, Z. 2023. Effectiveness of essential oils as inhibitors of quorum sensing activity. In: Renata, T., Andreja, L. P. and Domagoj, K. (eds.) *Power of microbes in industry and environment*. Croatia: Croatian Microbiological Society 110. URL:<http://oa.fins.uns.ac.rs/handle/123456789/253>

- D'Aquila, P., Sena, G., Crudo, M., Passarino, G., and Bellizzi, D. 2023. Effect of essential oils of apiaceae, lamiaceae, lauraceae, myrtaceae, and rutaceae family plants on growth, biofilm formation, and quorum sensing in *Chromobacterium violaceum*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis*. *Microorganisms* 11 (5):1150. doi:10.3390/microorganisms11051150.
- de Rapper, S., Kamatou, G., Viljoen, A., and van Vuuren, S. 2013. The *in vitro* antimicrobial activity of *Lavandula angustifolia* essential oil in combination with other aroma-therapeutic oils. *Evidence-Based Complementary and Alternative Medicine* 2013 (2013):852049. doi:10.1155/2013/852049.
- Dryden, M. S. 2010. Complicated skin and soft tissue infection. *Journal of Antimicrobial Chemotherapy* 65 (3):35-44. doi:10.1093/jac/dkq302.
- El-Tarabily, K. A., El-Saadony, M. T., Alagawany, M., Arif, M., Batiha, G. E., Khafaga, A. F., Elwan, H. A., Elnesr, S. S., and Abd El-Hack, M. E. 2021. Using essential oils to overcome bacterial biofilm formation and their antimicrobial resistance. *Saudi Journal of Biological Sciences* 28 (9):5145-5156. doi:10.1016/j.sjbs.2021.05.033.
- Ersanli, C., Tzora, A., Skoufos, I., Fotou, K., Maloupa, E., Grigoriadou, K., Voidarou, C., and Zeugolis, D. I. 2023. The assessment of antimicrobial and anti-biofilm activity of essential oils against *Staphylococcus aureus* strains. *Antibiotics* 12 (2):384. doi:10.3390/antibiotics12020384.
- EUCAST. 2022. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters.
- Fimbres-García, J. O., Flores-Sauceda, M., Othon-Díaz, E. D., García-Galaz, A., Tapia-Rodríguez, M. R., Silva-Espinoza, B. A., and Ayala-Zavala, J. F. 2022. Facing resistant bacteria with plant essential oils: Reviewing the oregano case. *Antibiotics (Basel)* 11 (12):1777. doi:10.3390/antibiotics11121777.

- Firmino, D. F., Cavalcante, T. T., Gomes, G. A., Firmino, N., Rosa, L. D., de Carvalho, M. G., and Catunda Jr, F. E. 2018. Antibacterial and antibiofilm activities of *Cinnamomum* sp. essential oil and cinnamaldehyde: antimicrobial activities. *The Scientific World Journal* 2018:ID 7405736 doi:10.1155/2018/7405736.
- Foster, T. J. 2017. Antibiotic resistance in *Staphylococcus aureus*: Current status and future prospects. *Federation of European Microbiological Societies Microbiology Reviews* 41 (3):430-449. doi:10.1093/femsre/fux007.
- Frydrysiak, E., Kunicka-Styczyńska, A., Śmigielski, K., and Frydrysiak, M. 2021. The impact of selected essential oils applied to non-woven viscose on bacteria that cause lower urinary tract infections—preliminary studies. *Molecules* 26 (22):6854. doi:10.3390/molecules26226854.
- Gallagher, J. 2013. Analysis: Antibiotic apocalypse BBC News website. URL: <https://www.bbc.com/news/health-21702647>.
- Ghannay, S., Aouadi, K., Kadri, A., and Snoussi, M. 2022. GC-MS profiling, vibriocidal, antioxidant, antibiofilm, and anti-quorum sensing properties of *Carum Carvi* l. essential oil: *In vitro* and *in silico* approaches. *Plants* 11 (8):1072. doi:10.3390/plants11081072.
- Ghavam, M., Bacchetta, G., Castangia, I., and Manca, M. L. 2022. Evaluation of the composition and antimicrobial activities of essential oils from four species of *Lamiaceae Martinov* native to Iran. *Scientific Reports* 12 (1):17044. doi:10.1038/s41598-022-21509-5.
- Greenberg, E. P. 2003. Bacterial communication and group behavior. *The Journal of Clinical Investigation* 112 (9):1288-1290. doi:10.1172/JCI20099.
- Hammer, K. A., Carson, C. F., and Riley, T. V. 2012. Effects of *Melaleuca alternifolia* (tea tree) essential oil and the major monoterpene component terpinen-4-ol on the development of single-and

multistep antibiotic resistance and antimicrobial susceptibility. *Antimicrobial Agents and Chemotherapy* 56 (2):909-915. doi:10.1128/aac.05741-11.

Hristova, Y., Gochev, V., Wanner, J., Jirovetz, L., Schmidt, E., and Girova, T. 2013. Chemical composition and antifungal activity of essential oil of *Salvia sclarea* L. from Bulgaria against clinical isolates of *Candida* species. *Journal of Bioscience and Biotechnology* 2 (1):39-44. ISSN:1314-6238.

Iseppi, R., Di Cerbo, A., Aloisi, P., Manelli, M., Pellesi, V., Provenzano, C., Camellini, S., Messi, P., and Sabia, C. 2020. *In vitro* activity of essential oils against planktonic and biofilm cells of extended-spectrum  $\beta$ -lactamase (ESBL)/carbapenamase-producing Gram-negative bacteria involved in human nosocomial infections. *Antibiotics (Basel)* 9 (5):272. doi:10.3390/antibiotics9050272.

Iseppi, R., Mariani, M., Benvenuti, S., Truzzi, E., and Messi, P. 2023. Effects of *Melaleuca alternifolia* Chell (Tea Tree) and *Eucalyptus globulus* Labill. essential oils on antibiotic-resistant bacterial biofilms. *Molecules* 28 (4):1671. doi:10.3390/molecules28041671.

Jafri, H., Husain, F. M., and Ahmad, I. 2014. Antibacterial and antibiofilm activity of some essential oils and compounds against clinical strains of *Staphylococcus aureus*. *Journal of Biomedical and Therapeutic Sciences* 1 (1):65-71.

URL:[https://d1wqtxts1xzle7.cloudfront.net/39544528/Antibacterial\\_and\\_antibiofilm\\_activity\\_o20151030-5750-rc230b-libre.pdf?1446189634=&response-content-](https://d1wqtxts1xzle7.cloudfront.net/39544528/Antibacterial_and_antibiofilm_activity_o20151030-5750-rc230b-libre.pdf?1446189634=&response-content-disposition=inline%3B+filename%3DAntibacterial_and_antibiofilm_activity_o.pdf&Expires=1711550140&Signature=Cbl-mJuxmZJGzDmtpZ~pTUcTArqtCkeBqPLENU2EJscxjuyN9ngBjfg80UWiUPLPMnwlihjEEaPAeUdy82dO0xv63j8uOorDk31dUgjtmfZZcl5lqdlIDwONuf47pcoBHainkyMDbpCsNyj4Qp9gwi8Rp6XNiaMDIYuogp9-GV3GSCPA8H9uEkva9m~fIY4Jrt6DUAIDjcyBwp0pisxOG4zsitWK46QIp934VGcvEEEWBplLKXLE)

[disposition=inline%3B+filename%3DAntibacterial\\_and\\_antibiofilm\\_activity\\_o.pdf&Expires=1711550140&Signature=Cbl-](https://d1wqtxts1xzle7.cloudfront.net/39544528/Antibacterial_and_antibiofilm_activity_o20151030-5750-rc230b-libre.pdf?1446189634=&response-content-disposition=inline%3B+filename%3DAntibacterial_and_antibiofilm_activity_o.pdf&Expires=1711550140&Signature=Cbl-mJuxmZJGzDmtpZ~pTUcTArqtCkeBqPLENU2EJscxjuyN9ngBjfg80UWiUPLPMnwlihjEEaPAeUdy82dO0xv63j8uOorDk31dUgjtmfZZcl5lqdlIDwONuf47pcoBHainkyMDbpCsNyj4Qp9gwi8Rp6XNiaMDIYuogp9-GV3GSCPA8H9uEkva9m~fIY4Jrt6DUAIDjcyBwp0pisxOG4zsitWK46QIp934VGcvEEEWBplLKXLE)

[mJuxmZJGzDmtpZ~pTUcTArqtCkeBqPLENU2EJscxjuyN9ngBjfg80UWiUPLPMnwlihjEEaPAeUdy82dO0xv63j8uOorDk31dUgjtmfZZcl5lqdlIDwONuf47pcoBHainkyMDbpCsNyj4Qp9gwi8Rp6XNiaMDIYuogp9-](https://d1wqtxts1xzle7.cloudfront.net/39544528/Antibacterial_and_antibiofilm_activity_o20151030-5750-rc230b-libre.pdf?1446189634=&response-content-disposition=inline%3B+filename%3DAntibacterial_and_antibiofilm_activity_o.pdf&Expires=1711550140&Signature=Cbl-mJuxmZJGzDmtpZ~pTUcTArqtCkeBqPLENU2EJscxjuyN9ngBjfg80UWiUPLPMnwlihjEEaPAeUdy82dO0xv63j8uOorDk31dUgjtmfZZcl5lqdlIDwONuf47pcoBHainkyMDbpCsNyj4Qp9gwi8Rp6XNiaMDIYuogp9-GV3GSCPA8H9uEkva9m~fIY4Jrt6DUAIDjcyBwp0pisxOG4zsitWK46QIp934VGcvEEEWBplLKXLE)

[GV3GSCPA8H9uEkva9m~fIY4Jrt6DUAIDjcyBwp0pisxOG4zsitWK46QIp934VGcvEEEWBplLKXLE](https://d1wqtxts1xzle7.cloudfront.net/39544528/Antibacterial_and_antibiofilm_activity_o20151030-5750-rc230b-libre.pdf?1446189634=&response-content-disposition=inline%3B+filename%3DAntibacterial_and_antibiofilm_activity_o.pdf&Expires=1711550140&Signature=Cbl-mJuxmZJGzDmtpZ~pTUcTArqtCkeBqPLENU2EJscxjuyN9ngBjfg80UWiUPLPMnwlihjEEaPAeUdy82dO0xv63j8uOorDk31dUgjtmfZZcl5lqdlIDwONuf47pcoBHainkyMDbpCsNyj4Qp9gwi8Rp6XNiaMDIYuogp9-GV3GSCPA8H9uEkva9m~fIY4Jrt6DUAIDjcyBwp0pisxOG4zsitWK46QIp934VGcvEEEWBplLKXLE)

tGferzqcIPpqrW3~iN7NWtV6DfBR4zbdO-

~f2i2~1le7BLdehsb8xE5AG6BlE51ZxZQ3L50jiw5tzW1Tx2M5E3IUGBxVVqRg\_\_&Key-Pair-

Id=APKAJLOHF5GGSLRBV4ZA.

Jansen, K. U., Gruber, W. C., Simon, R., Wassil, J., and Anderson, A. S. 2021. The impact of human vaccines on bacterial antimicrobial resistance. A review. *Environmental Chemistry Letters* 19 (6):4031-4062. doi:10.1007/s10311-021-01274-z.

Kafa, A. H. T., Aslan, R., Celik, C., and Hasbek, M. 2022. Antimicrobial synergism and antibiofilm activities of *Pelargonium graveolens*, *Rosemary officinalis*, and *Mentha piperita* essential oils against extreme drug-resistant *Acinetobacter baumannii* clinical isolates. *Zeitschrift für Naturforschung C* 77 (3-4):95-104. doi:10.1515/znc-2021-0079.

Kerekes, E. B., Vidács, A., Takó, M., Petkovits, T., Vágvölgyi, C., Horváth, G., Balázs, V. L., and Krisch, J. 2019. Anti-biofilm effect of selected essential oils and main components on mono-and polymicrobial bacterial cultures. *Microorganisms* 7 (9):345. doi:10.3390/microorganisms7090345.

Khalaf, Z. Z., and Zahra, L. A. 2020. Evaluation of the activity of essential oil and hydrosol from *Eucalyptus camaldulensis* against some bacterial species. *Iraqi Journal of Science* 61 (6):1282-1288. doi:10.24996/ij.s.2020.61.6.5.

Khosakueng, M., Taweekaisupapong, S., Boonyanugomol, W., Prapatpong, P., Wongkaewkhiaw, S., and Kanthawong, S. 2024. *Cymbopogon citratus* L. essential oil as a potential anti-biofilm agent active against antibiotic-resistant bacteria isolated from chronic rhinosinusitis patients. *Biofouling* 40 (1):26-39. doi:10.1080/08927014.2024.2305387.

Koçak, U. Ç., Arslan, E., Çobanoğlu, Ş., COŞKUN, M. K., YAZICI, A., and ÖRTUCU, S. 2022. The effect of nisin and chloramphenicol combination on *Staphylococcus aureus* ATCC 6538 biofilm

- structure. *Bilecik Şeyh Edebali Üniversitesi Fen Bilimleri Dergisi* 9 (2):713-720. doi:10.35193/bseufbd.1058558.
- Kong, A. S.-Y., Maran, S., Yap, P. S.-X., Lim, S.-H. E., Yang, S.-K., Cheng, W.-H., Tan, Y.-H., and Lai, K.-S. 2022. Anti-and pro-oxidant properties of essential oils against antimicrobial resistance. *Antioxidants* 11 (9):1819. doi:10.3390/antiox11091819.
- Křížkovská, B., Hoang, L., Brdová, D., Klementová, K., Szemerédi, N., Loučková, A., Kronusová, O., Spengler, G., Kaštánek, P., Hajšlová, J., Viktorová, J., and Lipov, J. 2023. Modulation of the bacterial virulence and resistance by well-known European medicinal herbs. *Journal of Ethnopharmacology* 312:116484. doi:10.1016/j.jep.2023.116484.
- Lahkimi, A., Bouchra, L., chaouch, m., Nechad, I., and Eloutassi, N. 2020. Antibacterial, antifungal and antioxidant activity of *Lavandula Angustifolia* of the middle atlas central (Morocco). *Moroccan Journal of Chemistry* 8 (4):8-4. doi:10.48317/IMIST.PRSM/morjchem-v8i4.18680.
- Lee, K., Lee, J.-H., Kim, S.-I., Cho, M. H., and Lee, J. 2014. Anti-biofilm, anti-hemolysis, and anti-virulence activities of black pepper, cananga, myrrha oils, and nerolidol against *Staphylococcus aureus*. *Applied Microbiology and Biotechnology* 98 (22):9447-9457. doi:10.1007/s00253-014-5903-4.
- Leigh-de Rapper, S., and van Vuuren, S. F. 2020. Odoriferous therapy: A review identifying essential oils against pathogens of the respiratory tract. *Chemistry & Biodiversity* 17 (6):e2000062. doi:10.1002/cbdv.202000062.
- Li, B., Qiu, Y., Shi, H., and Yin, H. 2016. The importance of lag time extension in determining bacterial resistance to antibiotics. *Analyst* 141 (10):3059-3067. doi:10.1039/C5AN02649K.
- Li, Y.-H., and Tian, X. 2012. Quorum sensing and bacterial social interactions in biofilms. *Sensors* 12 (3):2519-2538. doi:10.3390/s120302519.

- Liu, C., Cheng, F., Aisa, H. A., and Maiwulanjiang, M. 2023. Comprehensive study of components and antimicrobial properties of essential oil extracted from *Carum Carvi* l. seeds. *Antibiotics* 12 (3):591. doi:10.3390/antibiotics12030591.
- Locke, J. B., Hilgers, M., and Shaw, K. J. 2009. Novel ribosomal mutations in *Staphylococcus aureus* strains identified through selection with the oxazolidinones linezolid and torezolid (TR-700). *Antimicrobial Agents and Chemotherapy* 53 (12):5265-74. doi:10.1128/aac.00871-09.
- Mahumane, G. D. 2016. "Antimicrobial Activity and Chemical Analysis of *Eucalyptus Radiata* Leaf Essential Oil." Masters Dissertation, University of the Witwatersrand, Gauteng, South Africa.
- McMahon, M. A., Tunney, M. M., Moore, J. E., Blair, I. S., Gilpin, D. F., and McDowell, D. A. 2008. Changes in antibiotic susceptibility in *Staphylococci* habituated to sub-lethal concentrations of tea tree oil (*Melaleuca alternifolia*). *Letters in Applied Microbiology* 47 (4):263-268. doi:10.1111/j.1472-765X.2008.02420.x.
- Merghni, A., Haddaji, N., Bouali, N., Alabbosh, K. F., Adnan, M., Snoussi, M., and Noumi, E. 2022. Comparative study of antibacterial, antibiofilm, antismearing and anti-quorum sensing activities of *Origanum vulgare* essential oil and terpinene-4-ol against pathogenic bacteria. *Life* 12 (10):1616. doi:10.3390/life12101616.
- Merghni, A., Marzouki, H., Hentati, H., Aouni, M., and Mastouri, M. 2016. Antibacterial and antibiofilm activities of *Laurus nobilis* L. essential oil against *Staphylococcus aureus* strains associated with oral infections. *Current Research in Translational Medicine* 64 (1):29-34. doi:10.1016/j.patbio.2015.10.003.
- Moradi, F., Hadi, N., and Bazargani, A. 2020. Evaluation of quorum-sensing inhibitory effects of extracts of three traditional medicine plants with known antibacterial properties. *New Microbes and New Infections* 38:100769. doi:10.1016/j.nmni.2020.100769.

- Munita, J. M., and Arias, C. A. 2016. Mechanisms of antibiotic resistance. *In*: Kudva, I. T., Ames, I. A. and Zhang, Q. (eds.) *Virulence mechanisms of bacterial pathogens* Chile: Microbiology Spectrum 481-511. doi:10.1128/microbiolspec.VMBF-0016-2015.
- Naeem, A., Abbas, T., Ali, T. M., and Hasnain, A. 2018. Essential Oils: Brief background and uses. *Annals of Short Reports* 1 (1):1006. URL:[https://d1wqtxts1xzle7.cloudfront.net/82639569/essential-oils-brief-background-and-uses-516-libre.pdf?1648190476=&response-content-disposition=inline%3B+filename%3DEssential\\_Oils\\_Brief\\_Background\\_and\\_Uses.pdf&Expires=1711550777&Signature=WkgVLrBnrlwXEbuOxJO1zI3QAn1im3FHe5y2OV14~t8qyZsAnK-7P5crpFQEEFRTPVENTzmPLkRRtNV4lvJJr94br5dwQHGbFb8RzUno-xds-4k3PhFBKfHmLeG1trO9QBGGDpuT90vQID9SYLBY1I8SaasfHUlaXsDWmFWrxMNTLJN5yaxLg1BBIsDwnmMoXr8VOphElXtaAjTubQ4ep5eCk3O7frSnsbd0Xd-9arakFu0y-JCabDHR~AVRHJum9m45h991iJ9Ta3Npp303g4uuKXT3pGf~rzHX00MmJXSIGVyT0B6O648Jw eIPhsDYByOnL4TvhHvb1EI-2wTgQ\\_\\_&Key-Pair-Id=APKAJLOHF5GGSLRBV4ZA](https://d1wqtxts1xzle7.cloudfront.net/82639569/essential-oils-brief-background-and-uses-516-libre.pdf?1648190476=&response-content-disposition=inline%3B+filename%3DEssential_Oils_Brief_Background_and_Uses.pdf&Expires=1711550777&Signature=WkgVLrBnrlwXEbuOxJO1zI3QAn1im3FHe5y2OV14~t8qyZsAnK-7P5crpFQEEFRTPVENTzmPLkRRtNV4lvJJr94br5dwQHGbFb8RzUno-xds-4k3PhFBKfHmLeG1trO9QBGGDpuT90vQID9SYLBY1I8SaasfHUlaXsDWmFWrxMNTLJN5yaxLg1BBIsDwnmMoXr8VOphElXtaAjTubQ4ep5eCk3O7frSnsbd0Xd-9arakFu0y-JCabDHR~AVRHJum9m45h991iJ9Ta3Npp303g4uuKXT3pGf~rzHX00MmJXSIGVyT0B6O648Jw eIPhsDYByOnL4TvhHvb1EI-2wTgQ__&Key-Pair-Id=APKAJLOHF5GGSLRBV4ZA).
- Nelson, R. R. 2000. Selection of resistance to the essential oil of *Melaleuca alternifolia* in *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy* 45 (4):549-550. doi:10.1093/jac/45.4.549.
- Noumi, E., Merghni, A., M, M. A., Haddad, O., Akmadar, G., De Martino, L., Mastouri, M., Ceylan, O., Snoussi, M., Al-Sieni, A., and De Feo, V. 2018. *Chromobacterium violaceum* and *Pseudomonas aeruginosa* PAO1: Models for evaluating anti-quorum sensing activity of *Melaleuca alternifolia* essential oil and its main component terpinen-4-ol. *Molecules* 23 (10):2672. doi:10.3390/molecules23102672.
- O'Toole, G. A. 2011. Microtiter dish biofilm formation assay. *Journal of Visualized Experiments* (47). doi:10.3791/2437.

- Oh, S., Yun, W., Lee, J., Lee, C., Kwak, W., and Cho, J. 2017. Effects of essential oil (blended and single essential oils) on anti-biofilm formation of *Salmonella* and *Escherichia coli*. *Journal of Animal Science and Technology* 59 (1):4. doi:10.1186/s40781-017-0127-7.
- Oppenheimer-Shaanan, Y., Steinberg, N., and Kolodkin-Gal, I. 2013. Small molecules are natural triggers for the disassembly of biofilms. *Trends in Microbiology* 21 (11):594-601. doi:10.1016/j.tim.2013.08.005.
- Orchard, A., Sandasi, M., Kamatou, G. P. P., Viljoen, A., and van Vuuren, S. 2017b. The *in vitro* antimicrobial activity and chemometric modelling of 59 commercial essential oils against pathogens of dermatological relevance. *Chemistry and Biodiversity* 14 (1):e1600218. doi:10.1002/cbdv.201600218.
- Orchard, A., and van Vuuren, S. 2017. Commercial essential oils as potential antimicrobials to treat skin diseases. *Evidence-Based Complementary and Alternative Medicine* 2017:1-92. doi:10.1155/2017/4517971.
- Orchard, A., Van Vuuren, S., Viljoen, A., and Kamatou, G. 2018. The *in vitro* antimicrobial evaluation of commercial essential oils and their combinations against acne. *International journal of cosmetic science* 40 (3):226-243. doi:10.1111/ics.12456.
- Özfenerci, M., Bali, E. B., Erdönmez, D., and ÇALIŞKAN, U. K. 2022. Aromatherapeutic essential oils and their pharmaceutical combinations: Tools for inhibition of quorum sensing activity and biofilm formation of human pathogens. *Istanbul Journal of Pharmacy* 52 (1):54-63. doi:10.26650/IstanbulJPharm.2022.998774.
- Panda, S. K., Buroni, S., Swain, S. S., Bonacorsi, A., da Fonseca Amorim, E. A., Kulshrestha, M., da Silva, L. C. N., and Tiwari, V. 2022. Recent advances to combat ESKAPE pathogens with special reference to essential oils. *Frontiers in Microbiology* 13:1029098.

- Pekmezovic, M., Aleksic, I., Barac, A., Arsic-Arsenijevic, V., Vasiljevic, B., Nikodinovic-Runic, J., and Senerovic, L. 2016. Prevention of polymicrobial biofilms composed of *Pseudomonas aeruginosa* and pathogenic fungi by essential oils from selected *Citrus* species. *Federation of European Microbiological Societies Pathogens and Disease* 74 (8):ftw102. doi:10.1093/femspd/ftw102.
- Pereira, C., Larsson, J., Hjort, K., Elf, J., and Andersson, D. I. 2021. The highly dynamic nature of bacterial heteroresistance impairs its clinical detection. *Communications Biology* 4 (1):521. doi:10.1038/s42003-021-02052-x.
- Poli, J. P., Guinoiseau, E., de Rocca Serra, D., Sutour, S., Paoli, M., Tomi, F., Quilichini, Y., Berti, L., and Lorenzi, V. 2018. Anti-quorum sensing activity of 12 essential oils on *Chromobacterium violaceum* and specific action of *cis-cis-p*-menthenolide from *Corsican mentha suaveolens* ssp. *insularis*. *Molecules* 23 (9). doi:10.3390/molecules23092125.
- Prabuseenivasan, S., Jayakumar, M., and Ignacimuthu, S. 2006. *In vitro* antibacterial activity of some plant essential oils. *BMC Complementary and Alternative Medicine* 6 (1):39. doi:10.1186/1472-6882-6-39.
- Reichling, J. 2020. Anti-biofilm and virulence factor-reducing activities of essential oils and oil components as a possible option for bacterial infection control. *Planta Medica* 86 (08):520-537. doi:10.1055/a-1147-4671.
- Reuter, J., Merfort, I., and Schempp, C. M. 2010. Botanicals in dermatology: An evidence-based review. *American Journal of Clinical Dermatology* 11 (4):247-267. doi:10.2165/11533220-000000000-00000.
- Rolfe, M. D., Rice, C. J., Lucchini, S., Pin, C., Thompson, A., Cameron, A. D., Alston, M., Stringer, M. F., Betts, R. P., Baranyi, J., Peck, M. W., and Hinton, J. C. 2012. Lag phase is a distinct growth phase

- that prepares bacteria for exponential growth and involves transient metal accumulation. *Journal of Bacteriology* 194 (3):686-701. doi:10.1128/jb.06112-11.
- Rubegeta, E., Ahmad, A., Kamatou, G. P. P., Sandasi, M., Sommerlatte, H., and Viljoen, A. M. 2019. Headspace analysis, antimicrobial and anti-quorum sensing activities of seven selected African *Commiphora* species. *South African Journal of Botany* 122:522-528. doi:10.1016/j.sajb.2018.03.001.
- 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. *Journal of Intercultural Ethnopharmacology* 5 (3):212-8. doi:10.5455/jice.20160331064446.
- SAMF, S. A. M. A. 2016. *South African Medicines Formulary* Edited by Rossiter, D. 12th Edition ed. Pretoria, South Africa Health and Medical Publishing Group. ISBN:978-1-875098-31-6.
- Sanchez-Cid, C., Guironnet, A., Keuschnig, C., Wiest, L., Vulliet, E., and Vogel, T. M. 2022. Gentamicin at sub-inhibitory concentrations selects for antibiotic resistance in the environment. *International Society for Microbial Ecology Communications* 2 (1):29. doi:10.1038/s43705-022-00101-y.
- Sartorius, B., Gray, A. P., Weaver, N. D., Aguilar, G. R., Swetschinski, L. R., Ikuta, K. S., Mestrovic, T., Chung, E., Wool, E. E., and Han, C. 2024. The burden of bacterial antimicrobial resistance in the WHO African region in 2019: a cross-country systematic analysis. *The Lancet Global Health* 12 (2):e201-e216. doi:10.1016/S2214-109X(23)00539-9.
- Shukla, S., and Toleti, S. R. 2017. An improved crystal violet assay for biofilm quantification in 96-well micro-titre plate.e100214. doi:10.1101/100214.

- Sienkiewicz, M., Łysakowska, M., Denys, P., and Kowalczyk, E. 2012. The antimicrobial activity of thyme essential oil against multidrug resistant clinical bacterial strains. *Microbial drug resistance* 18 (2):137-148. doi:10.1089/mdr.2011.0080.
- Singh, L. 2022. "Essential oil-loaded polymeric film for antimicrobial application to a urinary catheter." Master of Pharmacy, Department of Pharmacy and Pharmacology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa.
- Singh, V. K., Vaish, M., Johansson, T. R., Baum, K. R., Ring, R. P., Singh, S., Shukla, S. K., and Moskovitz, J. 2015. Significance of four methionine sulfoxide reductases in *Staphylococcus aureus*. *PLoS one* 10 (2):e0117594. doi:10.1371/journal.pone.0117594.
- Speck, S., Wenke, C., Feßler, A. T., Kacza, J., Geber, F., Scholtzek, A. D., Hanke, D., Eichhorn, I., Schwarz, S., Rosolowski, M., and Truyen, U. 2020. Borderline resistance to oxacillin in *Staphylococcus aureus* after treatment with sub-lethal sodium hypochlorite concentrations. *Heliyon* 6 (6):e04070. doi:10.1016/j.heliyon.2020.e04070.
- Stojanović-Radić, Z., Dimitrijević, M., Aleksić, A., and Stanković, N. 2021. Anti-staphylococcal activity of *Thymus vulgaris* and *Origanum vulgare* essential oils: timelapse kinetics, antibiofilm activity and synergistic potential. *Biologica Nyssana* 12 (1):33. doi:10.5281/zenodo.5522981.
- Tello, A., Austin, B., and Telfer, T. C. 2012. Selective pressure of antibiotic pollution on bacteria of importance to public health. *Environmental Health Perspectives* 120 (8):1100-6. doi:10.1289/ehp.1104650.
- Tsao, S.-m., and Yin, M.-c. 2001. *In vitro* activity of garlic oil and four diallyl sulphides against antibiotic-resistant *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. *Journal of Antimicrobial Chemotherapy* 47 (5):665-670. doi:10.1093/jac/47.5.665.

- Van, N. T. B., Vi, O. T., Yen, N. T. P., Nhung, N. T., Cuong, N. V., Kiet, B. T., Hoang, N. V., Hien, V. B., Thwaites, G., Campell, J., Choisy, M., and Carrique-Mas, J. 2022. Minimum inhibitory concentrations of commercial essential oils against common chicken pathogenic bacteria and their relationship with antibiotic resistance. *Journal of Applied Microbiology* 132 (2):1025-1035. doi:10.1111/jam.15302.
- Vattem, D. A., Mihalik, K., Crixell, S. H., and McLean, R. J. 2007. Dietary phytochemicals as quorum sensing inhibitors. *Fitoterapia* 78 (4):302-310. doi:10.1016/j.fitote.2007.03.009.
- Vázquez-Sánchez, D., Cabo, M. L., and Rodríguez-Herrera, J. J. 2015. Antimicrobial activity of essential oils against *Staphylococcus aureus* biofilms. *Food Science and Technology International* 21 (8):559-570. doi:10.1177/1082013214553996.
- Willems, H. M., Xu, Z., and Peters, B. M. 2016. Polymicrobial biofilm studies: from basic science to biofilm control. *Current Oral Health Reports* 3 (1):36-44. doi:10.1007/s40496-016-0078-y.
- Xu, Z., Liang, Y., Lin, S., Chen, D., Li, B., Li, L., and Deng, Y. 2016. Crystal violet and XTT assays on *Staphylococcus aureus* biofilm quantification. *Current Microbiology* 73 (4):474-482. doi:10.1007/s00284-016-1081-1.
- Yang, S.-K., Tan, N.-P., Chong, C.-W., Abushelaibi, A., Lim, S.-H.-E., and Lai, K.-S. 2021. The missing piece: Recent approaches investigating the antimicrobial mode of action of essential oils. *Evolutionary Bioinformatics* 17:1176934320938391. doi:10.1177/11769343209383.
- 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* 8:6-14. doi:10.2174/1874285801408010006.

Yu, Z., Tang, J., Khare, T., and Kumar, V. 2020. The alarming antimicrobial resistance in ESKAPEE pathogens: Can essential oils come to the rescue? *Fitoterapia* 140:104433. doi:10.1016/j.fitote.2019.104433.

**SUPPLEMENTARY DATA:**

**Table S1. Overview of antimicrobial activity of essential oils selected.**

| Essential oil   | Main components  | Previous antimicrobial activity                  |                   |                               |
|---|--|--|-------------------|-------------------------------|
|   |  | Pathogen   | MIC               | Reference                     |
| <i>Allium sativum</i> L.<br>(garlic)                  | Not specified  | <i>P. aeruginosa</i> susceptible clinical strain | 0.02 mg/mL        | Tsao and Yin (2001)           |
|   |  | <i>P. aeruginosa</i> resistant clinical strain   | 0.01 - 0.02 mg/mL |                               |
|   |  | <i>K. pneumoniae</i> resistant clinical strain   | 0.03 mg/mL        |                               |
|   | Diallyl sulfide (57.10%) Diallyl trisulfide (19.50%)   | <i>P. aeruginosa</i> (ATCC 27853)                | 0.13 mg/mL        | Orchard et al. (2017b)        |
| <i>Carum carvi</i> L.<br>(caraway)                    | Limonene (34.00%)<br>Carvone (64.10%)  | Resistant <i>K. pneumoniae</i>                   | 1.00 mg/mL        | Singh (2022)                  |
| <i>Cinnamomum verum</i><br>J.Presl<br>(cinnamon leaf) | Cinnamaldehyde (61.50%)<br>β-Caryophyllene (6.80%)<br>Cinnamyl acetate (6.50%)<br>Eugenol (3.70%)<br>β-Phellandene (3.70%) | GMRSA (ATCC 33592)                               | 0.13 mg/mL        | Orchard et al. (2017b)        |
|   |  | <i>P. aeruginosa</i> (ATCC 27853)                | 0.50 mg/mL        |                               |
|   |  | <i>S. aureus</i> (ATCC 25923)                    | 0.25 mg/mL        |                               |
|   |  | MRSA (ATCC 43300)                                | 0.50 mg/mL        | Orchard et al. (2018)         |
| <i>Cinnamomum zeylanicum</i> Blume<br>(cinnamon bark) | Eugenol (78.40%)   | <i>P. aeruginosa</i> (ATCC 27853)                | 1.00 mg/mL        | Orchard et al. (2017b)        |
|   |  | <i>S. aureus</i> (ATCC 25923)                    | 1.00 mg/mL        |                               |
|   |  | MRSA (ATCC 43300)                                | 1.00 mg/mL        |                               |
|   |  | GMRSA (ATCC 33592)                               | 1.00 mg/mL        |                               |
|   | Eugenol (84.70%)<br>β-caryophyllene (6.80%)  | Resistant <i>K. pneumoniae</i>                   | 0.50 mg/mL        | Singh (2022)                  |
|   | Cinnamaldehyde (52.40%)<br>Benzaldehyde (12.31%)<br>Benzoic acid (8.20%)<br>Benzyl alcohol (2.23%)                         | <i>K. pneumoniae</i> (ATCC 15380)                | 3.20 mg/mL        | Prabuseenivasan et al. (2006) |
|   |  | <i>P. aeruginosa</i> (ATCC 27853)                | > 0.80 mg/mL      |                               |
|   |  | <i>S. aureus</i> (ATCC 25923)                    | 3.20 mg/mL        |                               |

| Essential oil   | Main components  | Previous antimicrobial activity  |             |   |
|---|--|--|-------------|---|
|   |  | Pathogen   | MIC         | Reference                                   |
|   | (E)cinnamaldehyde<br>Cinnamoyl (E)-acetate<br>Eugenol  | <i>P. aeruginosa</i><br>(ATCC 27853)   | 0.50 mg/mL  | Firmino et al.<br>(2018)                    |
|   |  | <i>S. aureus</i> (ATCC 6538)   | 0.50 mg/mL  |   |
| <i>Commiphora myrrha</i><br>Engl. (myrrh)                 | Furanoesudema-1,3-<br>diene (52.90%)<br>Lindestrene (15.80%)   | <i>P. aeruginosa</i><br>(ATCC 27853)   | 1.00 mg/mL  | Orchard et al.<br>(2017b)                   |
|   |  | <i>S. aureus</i> (ATCC 25923)  | 1.00 mg/mL  |   |
|   |  | MRSA (ATCC 43300)  | 0.50 mg/mL  |   |
|   |  | GMRSA (ATCC 33592)   | 0.25 mg/mL  |   |
| <i>Cymbopogon citratus</i><br>Stapf (lemongrass)          | Limonene (11.70%)<br>Neral (28.90%)<br>Geranial (42.70%)   | <i>P. aeruginosa</i><br>(ATCC 27853)   | 1.00 mg/mL  | Orchard et al.<br>(2017b)                   |
|   |  | <i>S. aureus</i> (ATCC 25923)  | 1.00 mg/mL  |   |
|   |  | MRSA (ATCC 43300)  | 1.00 mg/mL  |   |
|   |  | GMRSA (ATCC 33592)   | 0.75 mg/mL  |   |
|   | β-myrcene (57.52%)<br>Carveol (13.80%)<br>Citral (12.34%)<br>6-methyl-5-hepten-2-<br>one (8.08%)<br>α-pinene (2.65%)<br>3-penten-2-Ol<br>(2.27%) | <i>K. pneumoniae</i> KKU-<br>CRS1 isolated from<br>patients with chronic<br>rhinosinusitis | 0.10% v/v   | Khosakueng et al.<br>(2024)                 |
|   |  | <i>P. aeruginosa</i> KKU-CRS2<br>isolated from patients with<br>chronic rhinosinusitis     | 3.20% v/v   |   |
| <i>Cymbopogon martinii</i><br>(palmarosa)                 | Geraniol (80.70%)  | <i>P. aeruginosa</i><br>(ATCC 27853)   | 1.00 mg/mL  | Orchard et al.<br>(2017b)                   |
|   |  | <i>S. aureus</i> (ATCC 25923)  | 0.50 mg/mL  |   |
|   |  | GMRSA (ATCC 33592)   | 1.00 mg/mL  |   |
| <i>Ferula galbaniflua</i><br>Boiss. & Buhse<br>(galbanum) | α -Pinene (8.30%)<br>β-Pinene (54.30%)<br>d-3-Carene (7.80%)   | <i>P. aeruginosa</i><br>(ATCC 27853)   | 0.66 mg/mL  | Orchard et al.<br>(2017b)                   |
| <i>Foeniculum dulce</i> Mill.<br>(fennel)                 | trans-Anethol<br>(80.73%)  | GMRSA (ATCC 33592)   | 1.00 mg/mL  | Orchard et al.<br>(2018)                    |
|   |  | <i>P. aeruginosa</i><br>(ATCC 27853)   | 1.00 mg/mL  |   |
|   | trans-Anethol<br>(80.73%)  | <i>K. pneumoniae</i><br>(ATCC 13883)   | 0.01 mg/mL  | Leigh-de Rapper<br>and van Vuuren<br>(2020) |
| <i>Laurus nobilis</i> L.<br>(bay)                         | 1,8-cineole (up to<br>30.80%)<br>Methyl eugenol (15.2 -<br>15.60%)   | <i>S. aureus</i> (ATCC 6538)   | 31.25 mg/mL | Merghni et al.<br>(2016)                    |

| Essential oil                                   | Main components   | Previous antimicrobial activity   |                   |                         |
|---|---|---|-------------------|-------------------------|
|   |   | Pathogen  | MIC               | Reference               |
|   | $\alpha$ -terpinyl acetate (up to 14.50%)   |   |                   |                         |
|   | Eugenol (54.40%)<br>Myrcene (18.50%)<br>Chavicol (11.50%)                             | <i>P. aeruginosa</i> (ATCC 27853)   | 1.00 mg/mL        | Orchard et al. (2017b)  |
|   |   | <i>S. aureus</i> (ATCC 25923)   | 1.00 mg/mL        |                         |
|   |   | MRSA (ATCC 43300)   | 1.00 mg/mL        |                         |
| GMRSA (ATCC 33592)                              | 1.00 mg/mL  |   |                   |                         |
| <i>Lavandula angustifolia</i> Bubani (lavender) | Not specified   | <i>P. aeruginosa</i> (ATCC 27853)   | 1.60% v/v         | Özferenci et al. (2022) |
|   |   | <i>K. pneumoniae</i> (ATCC 700603)  | 0.40% v/v         |                         |
|   |   | <i>S. aureus</i> (ATCC 29213)   | 0.40% v/v         |                         |
|   | Linalool (38.74%)   | <i>S. aureus</i> CIP 483  | 0.36 mg/mL        | Lahkimi et al. (2020)   |
| <i>Litsea cubeba</i> Pers. (may chang)          | $\alpha$ -Citral (38.28%)<br>$\beta$ -Citral (29.29%)<br>Cinene (16.53%)              | <i>A. baumannii</i> (with $\beta$ -lactam resistance genes, sulfa resistance genes and tetracycline resistance genes) | 1.08 mg/mL        | Yang et al. (2021)      |
|   | Geranial (44.60%)<br>Neral (28.80%)   | <i>S. aureus</i> (ATCC 25923)   | 1.00 mg/mL        | Orchard et al. (2017b)  |
|   |   | MRSA (ATCC 43300)   | 0.50 mg/mL        |                         |
| GMRSA (ATCC 33592)                              | 0.50 mg/mL  |   |                   |                         |
| <i>Melaleuca alternifolia</i> Cheel (tea tree)  | Terpinen-4-ol (43.29%)<br>$\gamma$ -terpinene (20.16%)<br>$\alpha$ -terpinene (8.89%) | <i>S. aureus</i> (clinical isolate resistant to methicillin)  | 8.00 $\mu$ g/mL   | Iseppi et al. (2023)    |
|   |   | <i>E. faecium</i> (clinical isolate resistant to vancomycin)  | 64.00 $\mu$ g/mL  |                         |
|   | Not specified   | MDR <i>P. aeruginosa</i>  | 0.01 to 0.50% w/v | Bučková et al. (2018)   |
|   | Terpinen-4-ol<br>p-cymene   | <i>A. baumannii</i>   | 0.12 - 0.25% v/v  | Sakkas et al. (2016)    |
|   | 1,8-Cineole (5.20%)<br>$\gamma$ -Terpinene (19.50%)<br>Terpinen-4-ol (40.40%)         | <i>S. aureus</i> (ATCC 25923)   | 0.05 mg/mL        | Noumi et al. (2018)     |
| MRSA (ATCC 43300)                               |   | 0.05 mg/mL  |                   |                         |

| Essential oil                                    | Main components   | Previous antimicrobial activity  |                         |                                  |
|--|---|--|-------------------------|----------------------------------|
|  |   | Pathogen   | MIC                     | Reference                        |
|  | Not specified   | metallo-beta-lactamase (MBL)-producing <i>P. aeruginosa</i>                      | 16.00 µg/mL             | Iseppi et al. (2020)             |
|  | Not specified   | extended-spectrum β-lactamase (ESBL)-producing <i>K. pneumoniae</i>              | 16.00 µg/mL             |                                  |
|  | Terpinen-4-ol<br>α-terpinene<br>p-cymene<br>(tested against 10 different oils from different suppliers) | GMRSA (ATCC 33592)   | 0.50 - 2.50 mg/mL       | Brun et al. (2019)               |
|  |   | <i>P. aeruginosa</i> (ATCC BAA-2108)   | 0.25 - 2.00 mg/mL       |                                  |
|  | Not specified   | <i>A. baumannii</i> (ATCC 19606)   | 0.80% v/v               | Özfenerci et al. (2022)          |
|  |   | <i>K. pneumoniae</i> (ATCC 700603)   | 0.80% v/v               |                                  |
|  |   | <i>S. aureus</i> (ATCC 29213)  | 0.40% v/v               |                                  |
| <i>Matricaria recutita</i> L. (german chamomile) | Terpinen-4-ol (44.60%)<br>γ-Terpinene (16.60%)<br>p-Cymene (9.60%)                                      | <i>S. aureus</i> (ATCC 25923)  | 0.50 mg/mL              | Orchard et al. (2018)            |
|  |   | GMRSA (ATCC 33592)   | 0.25 mg/mL              |                                  |
|  |   | <i>P. aeruginosa</i> (ATCC 27853)  | 1.00 mg/mL              |                                  |
| <i>Ocimum tenuiflorum</i> L. (holy basil)        | Not specified   | <i>K. pneumoniae</i> KKU-CRS1 isolated from patients with chronic rhinosinusitis | 0.20 - 0.80% v/v        | Khosakueng et al. (2024)         |
|  |   | <i>P. aeruginosa</i> KKU-CRS2 isolated from patients with chronic rhinosinusitis | > 3.20% v/v             |                                  |
|  | Linalool (55.20%)<br>Eugenol (10.40%)   | <i>S. aureus</i> (ATCC 25923)  | 0.50 mg/mL              | Orchard et al. (2018)            |
|  |   | MRSA (ATCC 43300)  | 1.00 mg/mL              |                                  |
|  |   | MRSA (ATCC 33592)  | 0.25 mg/mL              |                                  |
|  |   | <i>P. aeruginosa</i> (ATCC 27853)  | 1.00 mg/mL              |                                  |
|  | <i>Origanum vulgare</i> L. (oregano)  | Thymol (78.21%)  | MDR <i>Enterobacter</i> | 0.31 ± 0.00 to 5.00 ± 0.00 µL/mL |

| Essential oil                                       | Main components   | Previous antimicrobial activity               |                      |                               |
|---|---|---|----------------------|-------------------------------|
|   |   | Pathogen                                      | MIC                  | Reference                     |
|   | Not specified   | MDR <i>P. aeruginosa</i>                      | 0.01 to 0.50% w/v    | Bučková et al. (2018)         |
|   |   | <i>P. aeruginosa</i>                          | 0.03% w/v            |                               |
|   |   | <i>E. cloacae</i>                             | 0.01% w/v            |                               |
|   | Guaiacol-p-vinil (68.67%)<br>p-Cymene (4.60%)<br>β-ionol(3.16%)   | <i>P. aeruginosa</i> PA01                     | 0.05 mg/mL           | Merghni et al. (2022)         |
|   |   | <i>S. aureus</i> (ATCC 6538)                  | 0.05 mg/mL           |                               |
| Carvacrol<br>Thymol                                 | <i>A. baumannii</i>   | 0.25 - 0.37% v/v                              | Sakkas et al. (2016) |                               |
| <i>Pelargonium graveolens</i> L'Hér (rose geranium) | Not specified   | <i>A. baumannii</i> clinical isolates (XDR-A) | 5.00 - 20.00 μl/mL   | Kafa et al. (2022)            |
|   | Not specified   | <i>K. pneumoniae</i> (ATCC 15380)             | 12.80 mg/mL          | Prabuseenivasan et al. (2006) |
|   |   | <i>P. aeruginosa</i> (ATCC 27853)             | > 12.80 mg/mL        |                               |
|   |   | <i>S. aureus</i> (ATCC 25923)                 | > 12.80 mg/mL        |                               |
|   | Citronellol (26.70%)<br>Eudesmol (12.50%)<br>Citronellyl formate (11.50%)<br>Menthone (7.80%)<br>Linalool (7.20%) | <i>S. aureus</i> (ATCC 25923)                 | 1.00 mg/mL           | Orchard et al. (2018)         |
|   |   | MRSA (ATCC 33592)                             | 0.25 mg/mL           |                               |
|   |   | <i>P. aeruginosa</i> (ATCC 27853)             | 1.00 mg/mL           |                               |
| <i>Pogostemon patchouli</i> Benth. (patchouli)      | β-Patchoulene (38.30%)<br>α-Bulnesene (13.00%)<br>α-Guaiene (11.90%)  | <i>P. aeruginosa</i> (ATCC 27853)             | 1.00 mg/mL           | Orchard et al. (2017b)        |
|   |   | <i>S. aureus</i> (ATCC 25923)                 | 0.50 mg/mL           |                               |
|   |   | MRSA (ATCC 43300)                             | 1.00 mg/mL           |                               |
|   |   | GMRSA (ATCC 33592)                            | 0.25 mg/mL           |                               |
|   |   | MRSA (ATCC 33592)                             | 0.25 mg/mL           | Orchard et al. (2018)         |
|   |   | <i>P. aeruginosa</i> (ATCC 27853)             | 1.00 mg/mL           |                               |
| <i>Rosa damascena</i> Mill. (rose otto)             | Not specified   | <i>P. aeruginosa</i> PA01                     | 0.013 - 0.10% v/v    | Bali et al. (2021)            |
|   |   | <i>S. aureus</i> (ATCC 25923)                 | 1.00 mg/mL           |                               |

| Essential oil  | Main components   | Previous antimicrobial activity      |                        |                          |
|--|---|--------------------------------------|------------------------|--------------------------|
|  |   | Pathogen                             | MIC                    | Reference                |
|  | Phenyl ethyl alcohol (63.90%)<br>Citronellol (14.60%)<br>Nerol (6.80%)<br>Geraniol (7.90%)  | MRSA (ATCC 43300)                    | 1.00 mg/mL             | Orchard et al. (2018)    |
|  |   | MRSA (ATCC 33592)                    | 0.50 mg/mL             |                          |
|  |   | <i>P. aeruginosa</i> (ATCC 27853)    | 1.00 mg/mL             |                          |
| <i>Santalum austrocaledonicum</i> Merr. & L.M.Perry (sandalwood) | $\alpha$ -Santalol (32.10%)<br>cis- $\alpha$ -Santalol (11.30%)<br>4,5,9,10-Dehydroisolongifolene (12.30%)                                    | <i>S. aureus</i> (ATCC 25923)        | 0.50 mg/mL             | Orchard et al. (2017b)   |
|  |   | MRSA (ATCC 43300)                    | 0.50 mg/mL             |                          |
|  |   | GMRSA (ATCC 33592)                   | 0.25 mg/mL             |                          |
| <i>Syzygium aromaticum</i> L. (clove)                            | Not specified   | Clinical strains of <i>S. aureus</i> | 0.80 - 1.60 $\mu$ g/mL | Jafri et al. (2014)      |
|  |   | <i>E. cloacae</i>                    | 0.05% w/v              | Buřková et al. (2018)    |
|  | Eugenol (81.90%)<br>Isoeugenol (13.10%)   | <i>P. aeruginosa</i> (ATCC 27853)    | 1.00 mg/mL             | Orchard et al. (2017b)   |
|  |   | <i>S. aureus</i> (ATCC 25923)        | 0.88 mg/mL             |                          |
|  |   | MRSA (ATCC 43300)                    | 1.00 mg/mL             |                          |
| GMRSA (ATCC 33592)   | 1.00 mg/mL  |                                      |                        |                          |
|  |   |                                      |                        |                          |
| <i>Thymus vulgaris</i> Willk. (thyme)                            | Thymol (18.90%)<br>c-Terpinene (7.20%)<br>p-Cymene (41.00%)   | <i>P. aeruginosa</i> (ATCC 27853)    | 1.00 mg/mL             | Orchard et al. (2017b)   |
|  |   | <i>S. aureus</i> (ATCC 25923)        | 1.00 mg/mL             |                          |
|  | Thymol<br>p-cymene<br>Linalool  | <i>A. baumannii</i>                  | 0.25 - 0.50% v/v       | Sakkas et al. (2016)     |
|  | Thymol (37.00-55.00%)<br>Carvacrol (0.50-5.50%)<br>Linalool (1.50-6.50%)<br>Terpinen-4-ol (0.10-2.50%)<br>Methyl carvacrol ether (0.05-1.50%) | <i>A. baumannii</i>                  | 0.50 mg/mL             | Frydrysiak et al. (2021) |

| Essential oil                                   | Main components  | Previous antimicrobial activity    |                 |                         |
|---|--|------------------------------------|-----------------|-------------------------|
|   |  | Pathogen                           | MIC             | Reference               |
|   | Thymol (18.90%)<br>c-terpinene (7.20%)<br>p-Cymene (41.00%)                | GMRSA (ATCC 33592)                 | 1.00 mg/mL      | Orchard et al. (2017b)  |
|   | Not specified  | <i>E. cloacae</i>                  | 0.03% w/v       | Bučková et al. (2018)   |
|   | Thymol (18.90%)<br>$\gamma$ -Terpinene (7.20%)<br>p-Cymene (41.00%)        | Resistance <i>K. pneumoniae</i>    | 0.50 mg/mL      | Singh (2022)            |
|   | Not specified  | <i>A. baumannii</i> (ATCC 19606)   | 0.04% v/v       | Özfenerci et al. (2022) |
|   |  | <i>K. pneumoniae</i> (ATCC 700603) | 0.08% v/v       |                         |
|   |  | <i>S. aureus</i> (ATCC 29213)      | 0.04% v/v       |                         |
|   | Not specified  | MBL-producing <i>P. aeruginosa</i> | 4.00 $\mu$ g/mL | Iseppi et al. (2020)    |
| Not specified                                   | extended-spectrum $\beta$ -lactamase (ESBL)-producing <i>K. pneumoniae</i> | 32.00 $\mu$ g/mL                   |                 |                         |
| <i>Vetiveria zizanioides</i><br>Stapf (vetiver) | $\beta$ -Vetirenene (8.80%)<br>Zizanol (12.80%)                            | <i>S. aureus</i> (ATCC 25923)      | 0.50 mg/mL      | Orchard et al. (2018)   |
|   |  | MRSA (ATCC 43300)                  | 0.50 mg/mL      |                         |
|   |  | GMRSA (ATCC 33592)                 | 0.13 mg/mL      |                         |
|   |  | <i>P. aeruginosa</i> (ATCC 27853)  | 1.00 mg/mL      |                         |

**Table S2. Abridged GC-MS for selected essential oils**

| <b>Essential oil</b>                        | <b>Common name</b>  | <b>GC-MS main constituents</b>   | <b>Supplier</b> |
|---|---------------------|--|-----------------|
| <i>Allium sativum</i> L.                    | Garlic              | Dially sulfide 57.10%<br>Diallyl trisulfide 19.50%   | Escentia        |
| <i>Carum carvi</i> L.                       | Caraway             | Carvone 64.10%<br>Limonene 34.00%  | Prana Monde     |
| <i>Cinnamomum verum</i> J.Presl             | Cinnamon            | E-cinnamaldehyde 56.97%<br>Eugenyl acetate 8.96%<br>Linalool 3.17%<br>Limonene 2.80%   | Prana Monde     |
| <i>Cinnamomum zeylanicum</i><br>Blume       | Cinnamon            | Eugenol 71.98%<br>Eugenyl acetate 4.70%<br>$\beta$ -caryophyllene 3.31%  | Scatters        |
| <i>Commiphora myrrha</i> Engl.              | Myrrh               | Furanoeudesma-1,3 diene 46.38%<br>Curzerene 21.99%<br>Lindestrene 4.99%<br>Acetyl-8,12-epoxygermacra-1,4-<br>7-11-tetraene 4.61%                                 | Prana Monde     |
| <i>Cymbopogon citratus</i> Stapf            | Lemongrass          | Geraniol 36.99%<br>Neral 29.67%<br>Acetate de geranyle 7.48%<br>Geraniol 7.31%   | Prana Monde     |
| <i>Cymbopogon martinii</i> Stapf            | Palmarosa           | Geraniol 77.80%<br>Acetate de deranyle 9.60%<br>Linalol 3.02%<br>$\beta$ -caryophyllene 2.13%  | Prana Monde     |
| <i>Ferula galbaniflua</i> Boiss. &<br>Buhse | Galbanum            | $\beta$ -Pinene 54.30%   | Prana Monde     |
| <i>Foeniculum dulce</i> Mill.               | Fennel              | ( <i>E</i> )-anethole 82%<br>$\beta$ -caryophyllene 12.4%<br>1,8 cineole 19.4%   | Prana Monde     |
| <i>Laurus nobilis</i> L.                    | Bay                 | 1,8-cineole 54.10%<br>Acetate de terphenyl 7.50%<br>Sabinene 5.45%<br>$\alpha$ -pinene 5.32%   | Prana Monde     |
| <i>Lavandula angustifolia</i><br>Bubani     | Lavender            | Acetate de linalyle 38.14%<br>Linalol 32.99%<br>$\beta$ -caryophyllene 3.90%<br>Cis- $\beta$ ocimene 2.99%<br>Trans- $\beta$ ocinnene 2.50%<br>1,8 cineole 0.80% | Prana Monde     |
| <i>Litsea cubeba</i> Pers.                  | May chang           | Geraniol 34.10%<br>Neral 32.79%<br>Limonene 14.27%   | Scatters        |
| <i>Matricaria recutita</i> L.               | German<br>chamomile | e-trans- $\beta$ -farnesene 45.73%<br>$\alpha$ -bisabolol 9.56%<br>Oxyde D' $\alpha$ -bisabolol 5.22%  | Prana Monde     |

| Essential oil  | Common name   | GC-MS main constituents   | Supplier          |
|--|---------------|---|-------------------|
| <i>Melaleuca alternifolia</i> Cheel                  | Tea tree      | Terpinen-4-ol 44.60%<br>$\gamma$ -Terpinene 16.60%<br>p-Cymene 9.60%  | Locally distilled |
| <i>Ocinum tenuiflorum</i> L.                         | Holy basil    | Eugenol 30.2%<br>$\beta$ -caryophyllene 27.9%<br>$\beta$ -elemene 18.7%   | Subtle Energies   |
| <i>Origanum vulgare</i> L.                           | Oregano       | Carvacrol 62.73%<br>$\delta$ -terpinene 9.89%<br>Thymol 5.36%<br>p-cymene 4.97%   | Prana Monde       |
| <i>Pelargonium graveolens</i><br>L'Hér               | Rose geranium | Citronellol 27.76%<br>Geraniol 14.09%<br>Citronellyl formate 10.32%<br>Isomethane 7.82%<br>Geranyl formate 4.66%        | Scatters          |
| <i>Pogostemon patchouli</i> Benth.                   | Patchouli     | Patchouli alcohol 30.80%<br>Delta-guai-ene 18.04%<br>$\alpha$ -guai-ene 14.98%<br>$\alpha$ -patchoulene 7.42%           | Prana Monde       |
| <i>Rosa damascena</i> Mill.                          | Rose otto     | Citronellol 34.06%<br>Geraniol 14.29%<br>Nonadecane C19 8.28%<br>Nerol 6.72%  | Prana Monde       |
| <i>Santalum austrocaledonicum</i>                    | Sandalwood    | 2- $\alpha$ trans bergamotol 42.26%<br>z- $\beta$ santalol 17.21%<br>Lanceol 10.08%<br>Epi- $\beta$ santalol 3.52%      | Prana Monde       |
| <i>Syzygium aromaticum</i> (L.)<br>Merr. & L.M.Perry | Clove         | Eugenol 70.67%<br>Eugenal acetate 19.21%<br>Caryophyllene 8.13%   | Scatters          |
| <i>Thymus vulgaris</i> Willk.                        | Thyme         | Thymol 46.61%<br>p-cymene 17.38%<br>$\gamma$ -terpinene 9.58%<br>Carvacrol 5.16%<br>Linalol 4.36%                       | Prana Monde       |
| <i>Vetiveria zizanioides</i> Stapf                   | Vetiver       | Acide khusenique 15.87%<br>Khusimol 7.93%<br>Isovalencenol 7.39%<br>$\alpha$ -vetivone 3.73%<br>$\beta$ -vetivone 2.67% | Prana Monde       |

## CHAPTER 4: OVERVIEW AND CONCLUSION

---

---

### 4.1. Study highlights

This study aimed to investigate the response of pathogens to EOs and to further investigate what impact EOs have on inducing resistance. The objectives of this study included four assays: first, the minimum inhibitory activity assay was done to screen the 23 EOs to select five EOs; second, the anti-QS assay quantified the percentage of violacein inhibition, using the macrodilution method; third, the biofilm inhibition assay employed the microtiter plate method using crystal violet to quantify biofilm inhibition percentage. Finally, the induction of resistance assay utilized the microdilution method, exposing the culture to sub-inhibitory concentrations of the EO, and determining the MIC after each exposure. Subsequently, the lowest concentration supporting growth was sub-cultured for the next passage, repeated over 20 passages. After each passage, the MIC of three conventional antibiotics was also determined. The patterns of the MIC of the EOs and antibiotics over these passages were compared, alongside evaluating each of the five oils' overall activity across all four assays. All objectives were completed within the publication (Chapter 3).

### 4.2. Integration of all four assays

To the best of my knowledge, there has not been a comprehensive investigation into the relationship between EOs with varying minimum inhibitory activity and resistance mechanisms, including QS, biofilm formation and induction of resistance, prior to this study. This association provides a better understanding of which EOs should be focused on in future AMR research, based on their likelihood of following the resistance trends highlighted in this study.

#### 4.2.1 Minimum inhibitory activity and anti-quorum sensing activity

*Carum carvi* and *C. myrrha* displayed moderate to poor minimum inhibitory activity, however, they exhibited comparable and notable anti-QS ( $\geq 70.00\%$ ) to the other three EOs. This demonstrates that despite certain essential oils exhibiting poor minimum inhibitory activities, they should not be disregarded, as they could possess notable anti-quorum sensing properties, this is supported by Ahmad et al. (2015) and Mahumane (2016). Based on the outcomes of this study, irrespective of whether an EO had noteworthy or poor inhibitory activity, it still exhibited anti-QS activity. This observation may be attributed to the five specific EOs chosen for this study. It would be of interest to further explore whether this pattern holds true across different EOs with varying inhibitory activity.

Future research efforts could delve into the potential correlation between anti-QS activity and the major compounds within EOs, considering their individual antimicrobial properties (Burt 2004, Bakkali et al. 2008). This correlation was proven in a study conducted by Alrashidi et al. (2022), who found that the major compound of *Pimenta dioica* L., eugenol, had a greater anti-QS activity than the EO itself when tested against *S. aureus* ATCC 6538.

Despite no obvious differences in the MQSIC between the EOs with varying minimum inhibitory activity, a trend was observed in the MQSBC that followed the same classification as the minimum inhibitory activity, with *T. vulgaris* and *O. vulgare* having the lowest MQSBC, followed by *C. carvi* with a much greater MQSBC. Both *M. recutita* and *C. myrrha* had the highest MQSBC, and they were both classified as having overall poor minimum inhibitory activity.

The optimal EO is expected to demonstrate concurrent inhibitory and anti-QS activities at comparable concentrations. This dual functionality aims to effectively inhibit both planktonic and sessile bacteria populations. Nonetheless, in circumstances where this similarity is not achieved, it is preferable for the MQSIC to be less than the MIC, *T. vulgaris* was one such oil that demonstrated this. An EO that obstructs QS activity at a higher concentration than

the MIC, would not be considered an ideal antimicrobial for combating AMR, since, according to principles of pharmacotherapy, the lowest possible concentration should be used to counteract bacterial growth (Streit et al. 2016). This concept can also be applied when analysing inhibitory activity and biofilm inhibition. All five EOs exhibited MQSIC values lower than the averaged MIC values reported in the minimum inhibitory assay, which is corroborated by other studies looking at the anti-QS activity of EOs (Khan et al. 2009, Alvarez et al. 2012, Mahumane 2016). These studies were similar concerning the methodology and the relationship observed between the MIC and MQSIC found in this study.

#### **4.2.2 Minimum inhibitory activity and biofilm inhibition**

*Origanum vulgare* and *T. vulgaris*, being the two oils chosen for their noteworthy minimum inhibitory activity, exhibited noteworthy biofilm inhibition ( $\geq 70.00\%$ ) at the lowest concentration ( $\geq 0.05$  mg/mL) tested. Furthermore, these two oils demonstrated biofilm inhibition at four different concentrations. *Carum carvi* displayed notable biofilm inhibition (90.61%) only at the highest concentration (4.00 mg/mL). The EOs with poor minimum inhibitory activity inhibited biofilm formation only at the two highest concentrations tested 2.00 and 4.00 mg/mL. It can be deduced, based on the findings of this study, that EOs exhibiting noteworthy MIC values against planktonic bacteria may also possess the ability to impede sessile growth. This effectiveness resulted in the inhibition of biofilm formation at lower concentrations and across a wider concentration range compared to EOs with moderate to poor MIC values. When comparing the lowest concentration that *O. vulgare* and *T. vulgaris* could notably inhibit biofilm formation, it was lower than the average MIC value of both these oils against *S. aureus* ATCC 6538 and lower than the average MIC value against all strains of the ESKAPE pathogens studied.

Shin et al. (2021) established that erythromycin prevented the growth of planktonic micro-organisms and biofilms equally. A different trend was observed in the results of ciprofloxacin, that being that the concentration preventing biofilm inhibition was almost four to eight times higher than the MIC value against *S. aureus* ATCC 6538 (concentration

preventing planktonic growth) (Shin et al. 2021). However, macrolides (erythromycin) and fluoroquinolones (ciprofloxacin) have different mechanisms of antimicrobial action which could account for differences in their inhibitory activity against planktonic versus sessile bacteria. However, when the biofilm inhibition potential of gentamicin was tested in another study, the results concluded that noteworthy activity was detected at four times the MIC of this conventional antibiotic (Ersanli et al. 2023). In this study, ciprofloxacin inhibited biofilm formation at a concentration equal to the MIC value against *S. aureus* ATCC 6538, but also two concentrations higher than the MIC value.

#### **4.2.3 Anti-quorum sensing and biofilm inhibition**

The observed decrease in biofilm formation may imply a potential diminishing effect exerted by the EOs on the QS process, likely through the inhibition of receptors and molecules crucial to this signalling pathway (D'Aquila et al. 2023). *Carum carvi*, displayed borderline activity in both these assays, as an essential oil with moderate minimum inhibitory activity. Consequently, it may not warrant extensive investigation in the context of AMR due to its limited potential against these three assays that significantly impact resistance. The concentration that produced anti-QS activity was similar to that of *O. vulgare* and similar to the biofilm percentage inhibition of *O. vulgare* and *T. vulgaris*. *Matricaria recutita* and *C. myrrha*, both inhibited QS and biofilm formation at higher concentrations and with relatively lower percentage inhibition. A study investigating the effect of several EOs' on anti-QS and biofilm inhibition was used to compare the relationship between these two resistance mechanisms (D'Aquila et al. 2023). *Foeniculum vulgare* and *O. vulgare* were two oils, found to exhibit similar results for both assays. However, the previously mentioned study used *C. violaceum* for both assays, which differs from this study, even though *C. violaceum* is a biomonitor strain. The intention of this study was to use a strain that is known for growing strong biofilms and to use the same strain used in the induction of resistance assay.

#### **4.2.4 Minimum inhibitory activity and induction of resistance**

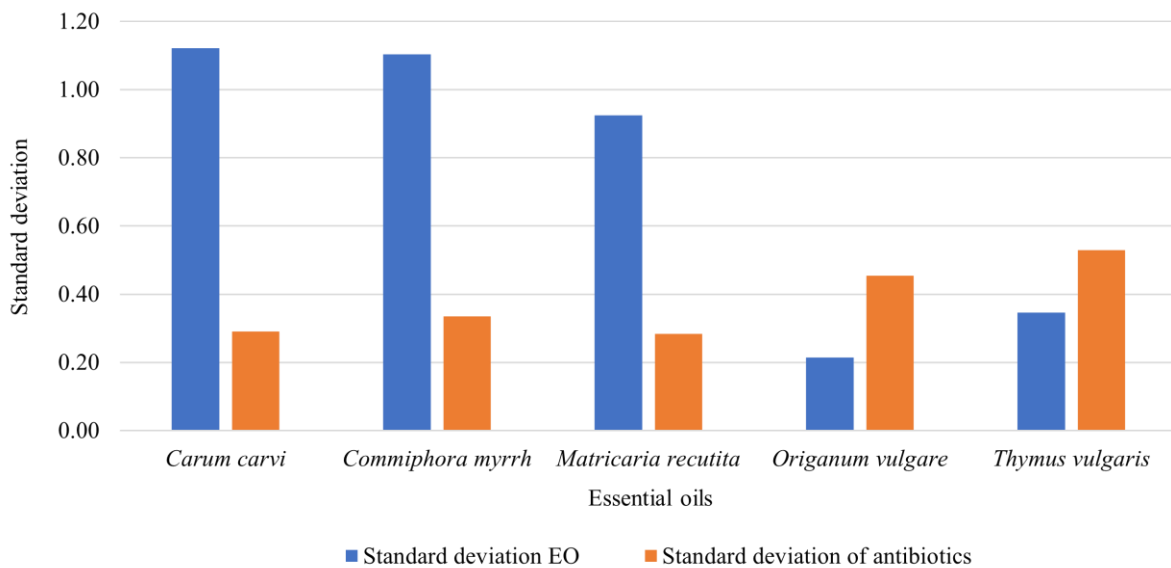
When analysing the consistency of antimicrobial activity of the EO in relation to the three conventional antibiotics a trend was observed, which is demonstrated in Figure 4.1. It was found that when *S. aureus* ATCC 6538 was exposed to the EOs with moderate and poor minimum inhibitory activity (*C. carvi*, *C. myrrha* and *M. recutita*), significant variance ( $SD \pm 1.12, \pm 1.10, \pm 0.92$ ) was observed in the MIC value of these three oils, over 20 passages of exposure. Contrary to these findings, *T. vulgaris* and *O. vulgare* had a substantial decrease in the variability of their MIC values, implying greater ability to sustain their inhibitory activity over subsequent exposure throughout the 20 passages.

Additionally, it was observed that exposure to *T. vulgaris* and *O. vulgare* led to increased variability in the MIC value of the three conventional antibiotics. Contrary to this, inconsistencies in MIC values of the antibiotics, due to exposure to the EOs with moderate to poor MIC values, were lower. This suggests that the overall degree of antimicrobial activity (MIC values) of EOs against the ESKAPE pathogens, does influence their ability to maintain their minimum inhibitory activity. However, it is important to note that this investigation focused on five specific EOs, and variations may arise when evaluating additional EOs. Furthermore, this relationship extends to influencing the impact of repeated exposure to oils with moderate to poor MIC values compared to oils with noteworthy MIC values, affecting MIC consistency and the effectiveness of conventional antibiotics.

#### **4.3 Future recommendations regarding antimicrobial resistance studies in essential oil research**

While anti-QS activity has been studied herein for the five selected EOs, data is lacking on many other EOs. In the past, antimicrobial activity was tested without QS being considered, however, in recent years both have been done (Camele et al. 2019, Alibi et al. 2020, Adeyemo et al. 2022, Alrashidi et al. 2022). Future recommendations should include gene sequencing to

understand specific mechanisms of action taking place whilst looking at the upregulation of virulent genes.



**Figure 4.1.** The comparison of consistency of the antimicrobial activity between the essential oils and the three conventional antibiotics (ciprofloxacin, erythromycin, and gentamicin) over 20 passages.

Fimbres-García et al. (2022) proposed that future research on new alternatives should include co-culture modelling experiments. This concept was introduced by Chan et al. (2018) whose findings demonstrated the upregulation of genes in *K. pneumoniae* and *A. baumannii* genes, including resistant ones, was due to the presence of another bacterial species. Consequently, the inclusion of these models in the assessment of essential oils will enable a more realistic understanding of the overall impact, given that infections typically comprise a multitude of bacterial species rather than a singular one. Although few studies testing this theory are evident, further research is warranted. Given the escalating levels of resistance, it is of utmost importance to contemplate various approaches, when contemplating the use of EOs, as new evidence becomes available on resistance (Kerekes et al. 2019).

The understanding of horizontal gene transfer in environmental and clinical contexts is primarily based on *in vitro* studies (Händel et al. 2014, Cen et al. 2020, Jiang et al. 2022, Michaelis and Grohmann 2023). A review recently published highlighted that cultivation under optimized conditions in liquid or solid media may not accurately reflect natural features, citing limitations such as the high cell density and the absence of a three-dimensional architecture present in biofilms (Michaelis and Grohmann 2023). In addition, *in vitro* models lack a host immune system found in natural biofilms. Bacterial cultivation on agar plates, while resembling biofilm growth to some extent, may exhibit fundamental differences from those observed in natural biofilms. These distinctions can arise from factors such as the lack of shear stress or variations in nutrient availability (Guzmán-Soto et al. 2021). Future studies on biofilms should incorporate the effect of EOs on the multistep biofilm formation process (Michaelis and Grohmann 2023). Furthermore, it was also revealed that sub-minimum inhibitory antibiotic concentrations can lead to antibiotic-induced stress and potentiate biofilm formation (Michaelis and Grohmann 2023). This was supported by two other studies investigating sub-inhibitory concentrations of antibiotics on biofilm inhibition (Bernardi et al. 2021, Mirzaei et al. 2022). This study also found that sub-inhibitory concentrations of the EO, potentiated biofilm inhibition. Therefore, this should be investigated further when applied to EOs as biofilm inhibitors.

*In vivo* studies play a crucial role in understanding the safety, efficacy, and pharmacokinetics of EOs for human application (Al-Harrasi et al. 2022). These studies help in exploring the absorption, distribution, metabolism, and excretion of EOs and their metabolites, providing essential insights into their bioavailability and pharmacological properties (Al-Harrasi et al. 2022). Furthermore, *in vivo* validation of EOs involves assessing their therapeutic potential, confirming their efficacy in treating various infections, and determining their impact on human health (Panda et al. 2020). By conducting *in vivo* studies, researchers can bridge the gap between *in vitro* findings and real-world applications, paving the way for the practical utilization of EOs as antimicrobial agents in the pharmaceutical industry (Dupuis et al. 2022, Chakraborty et al. 2023).

#### 4.4 Final remarks

In instances where pan-resistant pathogens are present, it is imperative to contemplate the necessity for a viable resolution. In such circumstances, the utilization of EOs may potentially function as such a solution, as it would minimize the problem of resistance to conventional antibiotics. It is hopeful that EOs are uniquely advantaged because of their chemical composition, which often includes a number of compounds, some with their own antimicrobial activity. This complexity makes it less likely for pathogens to develop resistance to EOs, as they would need to overcome a myriad of inhibitory actions. Unlike conventional antibiotics that often target specific pathways in bacteria.

The challenges associated with using EOs as alternatives to conventional antibiotics in AMR include the variability in composition due to factors like plant species, growth condition of plants and extraction methods, which affects consistency and reliability. Thus, standardization of variables previously mentioned, and quality control are imperative in future EO research. Currently, there is a shortage of robust clinical evidence since most studies are *in vitro* or *in vivo* rather than involving human participants. In addition to clinical studies, it is critical to understand the specific mechanisms of action and investigate potential interactions with conventional drugs.

A manual published in 1995 predicted a rise in the use of EOs, attributed to the increasing influence of environmentally conscious consumerism. This trend has led to greater utilization and development of plant-derived products (De Silva 1995, Nenaah et al. 2023). As expected, today EOs are commonly found in everyday items such as soaps, hand creams, sanitisers, medicinal ointments, and lip balms. Given the current high level of AMR, it is crucial to explore EOs as natural antimicrobial alternatives while prioritising research on their impact on resistance development. Therefore, this study was of utmost importance to identify the most potent and broad-spectrum antimicrobial EOs and then understand their effect on resistant emergence. This study provided compelling evidence supporting the superior inhibitory activity against MDR pathogens compared to conventional antibiotics. In particular, several

EOs notably inhibited ( $\leq 1.00$  mg/mL) a clinically multi-drug resistant strain of *A. baumannii*. Furthermore, these oils possessed the ability to inhibit resistant mechanisms such as QS and biofilm formation even at very low concentrations (sub-MICs). No instances of long-term resistance in *S. aureus* ATCC 6538 following repeated exposure to five essential oils were observed, making them a promising solution. *Thymus vulgaris* and *O. vulgare* were highlighted as highly favourable EOs in hindering multi-drug resistance pathogens, and resistance mechanisms (QS and biofilm formation) whilst not inducing resistance. The question imposed directly in the title of the thesis “Are essential oils a solution to combat antimicrobial resistance?” is affirmed and substantiated by the comprehensive evidence provided within this study, which unequivocally supports the notion that EOs indeed offer a viable solution to combat AMR.

## REFERENCES

---

---

- Adeyemo, R. O., Famuyide, I. M., Dzoyem, J. P., and Lyndy Joy, M. 2022. Anti-biofilm, antibacterial, and anti-quorum sensing activities of selected South African plants traditionally used to treat diarrhoea. *Evidence-Based Complementary and Alternative Medicine* 2022:1307801. doi:10.1155/2022/1307801.
- Aelenei, P., Miron, A., Trifan, A., Bujor, A., Gille, E., and Aprotosoai, A. C. 2016. Essential oils and their components as modulators of antibiotic activity against Gram-negative bacteria. *Medicines* 3 (3):19. doi:10.3390/medicines3030019.
- Agreles, M. A. A., Cavalcanti, I. D. L., and Cavalcanti, I. M. F. 2021. The role of essential oils in the inhibition of efflux pumps and reversion of bacterial resistance to antimicrobials. *Current Microbiology* 78 (10):3609-3619. doi:10.1007/s00284-021-02635-1.
- Ahmad, A., Viljoen, A. M., and Chenia, H. Y. 2015. The impact of plant volatiles on bacterial quorum sensing. *Letters in Applied Microbiology* 60 (1):8-19. doi:10.1111/lam.12343.
- Akram, F., Imtiaz, M., and Haq, I. u. 2023. Emergent crisis of antibiotic resistance: A silent pandemic threat to 21st century. *Microbial Pathogenesis* 174:105923. doi:10.1016/j.micpath.2022.105923.
- Al-Haidari, R. A., Shaaban, M. I., Ibrahim, S. R., and Mohamed, G. A. 2016. Anti-quorum sensing activity of some medicinal plants. *African Journal of Traditional, Complementary and Alternative Medicines* 13 (5):67-71. doi:10.21010/ajtcam.v13i5.10.
- Al-Harrasi, A., Bhatia, S., Ahmed, M. M., Anwer, K., and Sharma, P. 2022. Pharmacokinetics of essential oils. *Role of Essential Oils in the Management of COVID-19*. CRC Press 1:359-370. doi:10.1201/9781003175933.
- Al-Ouqaili, M. T. 2018. Biofilm antimicrobial susceptibility pattern for selected antimicrobial agents against planktonic and sessile cells of clinical isolates of *Staphylococci* using MICs, BICs and MBECs. *Asian Journal of Pharmaceutics* 12 (04):458001. doi:10.22377/AJP.V12I04.2938.

- Alfred Ngenge, T., Kucukaydin, S., Ceylan, O., and Duru, M. E. 2021. Evaluation of enzyme inhibition and anti-quorum sensing potentials of *Melaleuca alternifolia* and *Citrus sinensis* essential oils. *Natural Product Communications* 16 (9):1934578X211044565. doi:10.1177/1934578X211044565.
- Ali Mohammed, M. M., Nerland, A. H., Al-Haroni, M., and Bakken, V. 2013. Characterization of extracellular polymeric matrix, and treatment of *Fusobacterium nucleatum* and *Porphyromonas gingivalis* biofilms with DNase I and proteinase K. *Journal of Oral Microbiology* 5 (1):20015. doi:10.3402/jom.v5i0.20015.
- Alibi, S., Ben Selma, W., Ramos-Vivas, J., Smach, M. A., Touati, R., Boukadida, J., Navas, J., and Ben Mansour, H. 2020. Anti-oxidant, antibacterial, anti-biofilm, and anti-quorum sensing activities of four essential oils against multidrug-resistant bacterial clinical isolates. *Current Research in Translational Medicine* 68 (2):59-66. doi:10.1016/j.retram.2020.01.001.
- Alrashidi, A., Noumi, E., Snoussi, M., and Feo, V. 2022. Chemical composition, antibacterial and anti-quorum sensing activities of *Pimenta dioica* L. essential oil and its major compound (eugenol) against foodborne pathogenic bacteria. *Plants (Basel)* 11 (4):540. doi:10.3390/plants11040540.
- Álvarez-Martínez, F., Barrajon-Catalán, E., Herranz-López, M., and Micol, V. 2021. Antibacterial plant compounds, extracts and essential oils: An updated review on their effects and putative mechanisms of action. *Phytomedicine* 90:153626. doi:10.1016/j.phymed.2021.153626.
- Alvarez, M. V., Moreira, M. R., and Ponce, A. 2012. Antiquorum sensing and antimicrobial activity of natural agents with potential use in food. *Journal of Food Safety* 32 (3):379-387. doi:10.1111/j.1745-4565.2012.00390.x.
- Amin, M., Akrami, S., Haghparasty, F., and Hakimi, A. 2023. *In vitro* antibacterial activities of essential oils and extracts of six herbals against Gram-positive and Gram-negative bacteria. *Toxicology and Environmental Health Sciences* 15 (1):53-60. doi:10.1007/s13530-022-00160-7.

- Andersson, D. I., Nicoloff, H., and Hjort, K. 2019. Mechanisms and clinical relevance of bacterial heteroresistance. *Nature Reviews Microbiology* 17 (8):479-496. doi:10.1038/s41579-019-0218-1.
- Avershina, E., Khezri, A., and Ahmad, R. 2023. Clinical diagnostics of bacterial infections and their resistance to antibiotics—current state and whole genome sequencing implementation perspectives. *Antibiotics* 12 (4):781. doi:10.3390/antibiotics12040781.
- Bai, J., and Vittal, R. R. 2014. Quorum sensing inhibitory and anti-biofilm activity of essential oils and their *in vivo* efficacy in food systems. *Food Biotechnology* 28 (3):269-292. doi:10.1080/08905436.2014.932287.
- Bakkali, F., Averbeck, S., Averbeck, D., and Idaomar, M. 2008. Biological effects of essential oils—a review. *Food and chemical toxicology* 46 (2):446-475. doi:10.1016/j.fct.2007.09.106.
- Bali, E. B., Erdönmez, D., Yavuz, M., and Koca, U. 2021. Inhibitory effects of aromaterapeutic rose essential oil against bacterial quorum sensing and biofilm formation. *Journal of the Turkish Microbiology Society* 41 (3):245-253. doi:10.5222/TMCD.2021.72792.
- Batista, A. H. M., Moreira, A. C. D., de Carvalho, R. M., Sales, G. W. P., Nogueira, P. C. N., Grangeiro, T. B., Medeiros, S. C., Silveira, E. R., and Nogueira, N. A. P. 2017. Antimicrobial effects of violacein against planktonic cells and biofilms of *Staphylococcus aureus*. *Molecules* 22 (10):1534. doi:10.3390/molecules22101534.
- Becerril, R., Nerin, C., and Gomez-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. doi:10.1089/fpd.2011.1097.
- Bektas, S., Özdal, M., and Gürkök, S. 2023. Determination of antibacterial and antibiofilm activities for laurel (*Laurus nobilis* L.) essential oil against the fish pathogen *Pseudomonas* species. *Menba Kastamonu Üniversitesi Su Ürünleri Fakültesi Dergisi* 9 (1):25-33. doi:10.58626/menba.1289033.
- Benameur, Q., Gervasi, T., Pellizzeri, V., Pľuchtová, M., Grul'ová, D., Cicero, N., and Meriem-Hind, B. 2022. Comparison of sensitivity to a commercial *Origanum vulgare* essential oil between extended-spectrum  $\beta$ -lactamases (ESBL-) and non-ESBL-producing

- Enterobacteriaceae* isolates. *Natural Product Research* 36 (11):2830-2835. doi:10.1080/14786419.2021.1933969.
- Benkwitz-Bedford, S., Palm, M., Demirtas, T. Y., Mustonen, V., Farewell, A., Warringer, J., Parts, L., and Moradigaravand, D. 2021. Machine learning prediction of resistance to subinhibitory antimicrobial concentrations from *Escherichia coli* genomes. *Msystems* 6 (4):10.1128/msystems.00346-21. doi:10.1128/msystems.00346-21.
- Bernardi, S., Anderson, A., Macchiarelli, G., Hellwig, E., Cieplik, F., Vach, K., and Al-Ahmad, A. 2021. Subinhibitory antibiotic concentrations enhance biofilm formation of clinical *Enterococcus faecalis* isolates. *Antibiotics* 10 (7):874. doi:10.3390/antibiotics10070874.
- Bikels-Goshen, T., Landau, E., Saguy, S., and Shapira, R. 2010. *Staphylococcal* strains adapted to epigallocatechin gallate (EGCG) show reduced susceptibility to vancomycin, oxacillin and ampicillin, increased heat tolerance, and altered cell morphology. *International journal of food microbiology* 138 (1-2):26-31. doi:10.1016/j.ijfoodmicro.2010.01.011.
- Boskovic, M., Zdravkovic, N., Ivanovic, J., Janjic, J., Djordjevic, J., Starcevic, M., and Baltic, M. Z. 2015. Antimicrobial activity of thyme (*Thymus vulgaris*) and oregano (*Origanum vulgare*) essential oils against some food-borne microorganisms. *Procedia Food Science* 5:18-21. doi:10.1016/j.profoo.2015.09.005.
- Brentini Santiago, M., da Silva Moraes, T., Massuco, J., Silva, L., Lucarini, R., da Silva, D., Manzini Vieira, T., Miller Crotti, A., and Gomes Martins, C. 2018. *In vitro* evaluation of essential oils for potential antibacterial effects against *Xyllela fastidiosa*. *Journal of Phytopathology* 166:790-798. doi:10.1111/jph.12762.
- Brun, P., Bernabè, G., Filippini, R., and Piovan, A. 2019. *In vitro* antimicrobial activities of commercially available tea tree (*Melaleuca alternifolia*) essential oils. *Current microbiology* 76:108-116. doi:10.1007/s00284-018-1594-x.
- Bučková, M., Puškárová, A., Kalászová, V., Kisová, Z., and Pangallo, D. 2018. Essential oils against multidrug resistant Gram-negative bacteria. *Biologia* 73 (8):803-808. doi:10.2478/s11756-018-0090-x.

- Budri, P. E., Silva, N. C., Bonsaglia, E. C., Fernandes Júnior, A., Araújo Júnior, J. P., Doyama, J. T., Gonçalves, J. L., Santos, M. V., Fitzgerald-Hughes, D., and Rall, V. L. 2015. Effect of essential oils of *Syzygium aromaticum* and *Cinnamomum zeylanicum* and their major components on biofilm production in *Staphylococcus aureus* strains isolated from milk of cows with mastitis. *Journal of Dairy Science* 98 (9):5899-904. doi:10.3168/jds.2015-9442.
- Burt, S. 2004. Essential oils: their antibacterial properties and potential applications in foods- A review. *International Journal of Food Microbiology* 94 (3):223-53. doi:10.1016/j.ijfoodmicro.2004.03.022.
- Cáceres, M., Hidalgo, W., Stashenko, E., Torres, R., and Ortiz, C. 2020. Essential oils of aromatic plants with antibacterial, anti-biofilm and anti-quorum sensing activities against pathogenic bacteria. *Antibiotics* 9 (4):147. doi:10.3390/antibiotics9040147.
- Camele, I., Elshafie, H. S., Caputo, L., and De Feo, V. 2019. Anti-quorum sensing and antimicrobial effect of mediterranean plant essential oils against phytopathogenic bacteria. *Frontiers in Microbiology* 10:490001. doi:10.3389/fmicb.2019.02619.
- Carvalho, F., Coimbra, A. T., Silva, L., Duarte, A. P., and Ferreira, S. 2023. *Melissa officinalis* essential oil as an antimicrobial agent against *Listeria monocytogenes* in watermelon juice. *Food Microbiology* 109:104105. doi:10.1016/j.fm.2022.104105.
- Cen, T., Zhang, X., Xie, S., and Li, D. 2020. Preservatives accelerate the horizontal transfer of plasmid-mediated antimicrobial resistance genes via differential mechanisms. *Environment International* 138:105544. doi:10.1016/j.envint.2020.105544.
- Chaftar, N., Girardot, M., Labanowski, J. m., Ghrairi, T., Hani, K., Frere, J., and Imbert, C. 2016. Comparative evaluation of the antimicrobial activity of 19 essential oils. *Advances in microbiology, infectious diseases and public health* 2:1-15. doi:10.1007/5584\_2015\_5011.
- Chakraborty, M., Bardhan, T., Basu, M., and Bhattacharjee, B. 2022. Influence of sub-inhibitory dosage of cefotaxime on multidrug resistant *Staphylococcus haemolyticus* isolated from sick neonatal care unit. *Antibiotics* 11 (3):360. doi:10.3390/antibiotics11030360.

- Chakraborty, S., Dhibar, M., Das, A., Swain, K., and Pattnaik, S. 2023. Biological potential of essential oils: evaluation strategies. Edited by Inamuddin, *Essential Oils: Extraction Methods and Applications*: Scrivener Publishing LLC 23:521-549. doi:10.1002/9781119829614.ch23.
- Chan, A. P., Choi, Y., Brinkac, L. M., Krishnakumar, R., DePew, J., Kim, M., Hinkle, M. K., Lesho, E. P., and Fouts, D. E. 2018. Multidrug resistant pathogens respond differently to the presence of co-pathogen, commensal, probiotic and host cells. *Scientific Reports* 8 (1):8656. doi:10.1038/s41598-018-26738-1.
- Chao, S. C., Young, D. G., and Oberg, C. J. 2000. Screening for inhibitory activity of essential oils on selected bacteria, fungi and viruses. *Journal of Essential Oil Research* 12 (5):639 - 649. doi:10.1080/10412905.2000.9712177.
- Chouhan, S., Sharma, K., and Guleria, S. 2017. Antimicrobial activity of some essential oils—present status and future perspectives. *Medicines* 4 (3):58. doi:10.3390/medicines4030058.
- Ćirić, A. D., Petrović, J. D., Glamočlija, J. M., Smiljković, M. S., Nikolić, M. M., Stojković, D. S., and Soković, M. D. 2019. Natural products as biofilm formation antagonists and regulators of quorum sensing functions: A comprehensive review update and future trends. *South African Journal of Botany* 120:65-80. doi:10.1016/j.sajb.2018.09.010.
- Čobanović, R., Čabarkapa, I., Kokić, B., Rakita, S., and Tomičić, Z. 2023. Effectiveness of essential oils as inhibitors of quorum sensing activity. In: Renata, T., Andreja, L. P. and Domagoj, K. (eds.) *Power of microbes in industry and environment*. Croatia: Croatian Microbiological Society 46:110. ISBN:9537778193.
- Coşeriu, R. L., Vintilă, C., Pribac, M., Mare, A. D., Ciurea, C. N., Togănel, R. O., Cighir, A., Simion, A., and Man, A. 2023. Antibacterial effect of 16 essential oils and modulation of *mex* efflux pumps gene expression on multidrug-resistant *Pseudomonas aeruginosa* clinical isolates: Is cinnamon a good fighter? *Antibiotics* 12 (1):163. doi:10.3390/antibiotics12010163.
- Craft, K. M., Nguyen, J. M., Berg, L. J., and Townsend, S. D. 2019. Methicillin-resistant *Staphylococcus aureus* (MRSA): antibiotic-resistance and the biofilm phenotype. *Medicinal Chemistry Communications* 10 (8):1231-1241. doi:10.1039/c9md00044e.

- D'Aquila, P., Sena, G., Crudo, M., Passarino, G., and Bellizzi, D. 2023. Effect of essential oils of apiaceae, lamiaceae, lauraceae, myrtaceae, and rutaceae family plants on growth, biofilm formation, and quorum sensing in *Chromobacterium violaceum*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis*. *Microorganisms* 11 (5):1150. doi:10.3390/microorganisms11051150.
- Dalio, R. J., Maximo, H. J., Roma-Almeida, R., Barretta, J. N., José, E. M., Vitti, A. J., Blachinsky, D., Reuveni, M., and Pascholati, S. F. 2020. Tea tree oil induces systemic resistance against *Fusarium* wilt in banana and *Xanthomonas* infection in tomato plants. *Plants* 9 (9):1137. doi:10.3390/plants9091137.
- De Silva, T. 1995. A manual on the essential oil industry. In: Silva, K. T. D., ed. UNIDO workshop on essential oil and aroma chemical industries (3rd: 1995: Anadolu Universitesi) TBAM-ICS/UNIDO Training course on quality improvement of essential oils, Vienna, Austria. United Nations Industrial Development Organization., 222. ISBN:9789211064377.
- dos Santos Rodrigues, J. B., de Carvalho, R. J., de Souza, N. T., de Sousa Oliveira, K., Franco, O. L., Schaffner, D., de Souza, E. L., and Magnani, M. 2017. Effects of oregano essential oil and carvacrol on biofilms of *Staphylococcus aureus* from food-contact surfaces. *Food Control* 73:1237-1246. doi:10.1016/j.foodcont.2016.10.043.
- Dupuis, V., Cerbu, C., Witkowski, L., Potarniche, A.-V., Timar, M. C., Żychska, M., and Sabliov, C. M. 2022. Nanodelivery of essential oils as efficient tools against antimicrobial resistance: A review of the type and physical-chemical properties of the delivery systems and applications. *Drug Delivery* 29 (1):1007-1024. doi:10.1080/10717544.2022.2056663.
- Ebani, V. V., Pieracci, Y., Cagnoli, G., Bertelloni, F., Munafò, C., Nardoni, S., Pistelli, L., and Mancianti, F. 2023. *In vitro* antimicrobial activity of *Thymus vulgaris*, *Origanum vulgare*, *Satureja montana* and their mixture against clinical isolates responsible for canine otitis externa. *Veterinary Sciences* 10 (1):30. doi:10.3390/vetsci10010030.
- El-Tarabily, K. A., El-Saadony, M. T., Alagawany, M., Arif, M., Batiha, G. E., Khafaga, A. F., Elwan, H. A., Elnesr, S. S., and Abd El-Hack, M. E. 2021. Using essential oils to

- overcome bacterial biofilm formation and their antimicrobial resistance. *Saudi Journal of Biological Sciences* 28 (9):5145-5156. doi:10.1016/j.sjbs.2021.05.033.
- Elbestawy, M. K., El-Sherbiny, G. M., and Moghannem, S. A. 2023. Antibacterial, antibiofilm and anti-inflammatory activities of eugenol clove essential oil against resistant *Helicobacter pylori*. *Molecules* 28 (6):2448. doi:10.3390/molecules28062448.
- Elshafie, H. S., and Camele, I. 2017. An overview of the biological effects of some Mediterranean essential oils on human health. *BioMed Research International* 2017:9268468. doi:10.1155/2017/9268468.
- Elshafie, H. S., Mancini, E., Camele, I., De Martino, L., and De Feo, V. 2015. *In vivo* antifungal activity of two essential oils from Mediterranean plants against postharvest brown rot disease of peach fruit. *Industrial Crops and Products* 66:11-15. doi:10.1016/j.indcrop.2014.12.031.
- Eris, R., and Ulusoy, S. 2013. Rose, clove, chamomile essential oils and pine turpentine inhibit quorum sensing in *Chromobacterium violaceum* and *Pseudomonas aeruginosa*. *Journal of Essential Oil Bearing Plants* 16 (2):126-135. doi:10.1080/0972060X.2013.794026.
- Ersanli, C., Tzora, A., Skoufos, I., Fotou, K., Maloupa, E., Grigoriadou, K., Voidarou, C., and Zeugolis, D. I. 2023. The assessment of antimicrobial and anti-biofilm activity of essential oils against *Staphylococcus aureus* strains. *Antibiotics* 12 (2):384. doi:10.3390/antibiotics12020384.
- Fekry, M., Yahya, G., Osman, A., Al-Rabia, M. W., Mostafa, I., and Abbas, H. A. 2022. GC-MS analysis and microbiological evaluation of caraway essential oil as a virulence attenuating agent against *Pseudomonas aeruginosa*. *Molecules* 27 (23):8532. doi:10.3390/molecules27238532.
- Fimbres-García, J. O., Flores-Sauceda, M., Othon-Díaz, E. D., García-Galaz, A., Tapia-Rodríguez, M. R., Silva-Espinoza, B. A., and Ayala-Zavala, J. F. 2022. Facing resistant bacteria with plant essential oils: Reviewing the oregano case. *Antibiotics (Basel)* 11 (12):1777. doi:10.3390/antibiotics11121777.
- Firmino, D. F., Cavalcante, T. T., Gomes, G. A., Firmino, N., Rosa, L. D., de Carvalho, M. G., and Catunda Jr, F. E. 2018. Antibacterial and antibiofilm activities of *Cinnamomum* sp.

- essential oil and cinnamaldehyde: antimicrobial activities. *The Scientific World Journal* 2018:7405736. doi:10.1155/2018/7405736.
- Frydrysiak, E., Kunicka-Styczyńska, A., Śmigielski, K., and Frydrysiak, M. 2021. The impact of selected essential oils applied to non-woven viscose on bacteria that cause lower urinary tract infections—preliminary studies. *Molecules* 26 (22):6854. doi:10.3390/molecules26226854.
- Ghannay, S., Aouadi, K., Kadri, A., and Snoussi, M. 2022. GC-MS profiling, vibriocidal, antioxidant, antibiofilm, and anti-quorum sensing properties of *Carum Carvi* l. essential oil: *In vitro* and *in silico* approaches. *Plants* 11 (8):1072. doi:10.3390/plants11081072.
- Ghosh, S., Lahiri, D., Nag, M., Dey, A., Pandit, S., Sarkar, T., Pati, S., Abdul Kari, Z., Ishak, A. R., Edinur, H. A., and Ray, R. R. 2022. Phytocompound mediated blockage of quorum sensing cascade in ESKAPE pathogens. *Antibiotics (Basel)* 11 (1):61. doi:10.3390/antibiotics11010061.
- 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. doi:10.4161/viru.22507.
- Gustafson, E., Sean D. Cox Yoon C. Liew S. Grant Wyllie John R. Warmington John. 2001. The bacterial multiple antibiotic resistant (mar) phenotype leads to increased tolerance to tea tree oil. *Pathology* 33 (2):211-215. doi:10.1080/00313020123909.
- Guzmán-Soto, I., McTiernan, C., Gonzalez-Gomez, M., Ross, A., Gupta, K., Suuronen, E. J., Mah, T. F., Griffith, M., and Alarcon, E. I. 2021. Mimicking biofilm formation and development: Recent progress in *in vitro* and *in vivo* biofilm models. *iScience* 24 (5):102443. doi:10.1016/j.isci.2021.102443.
- Hakimi Alni, R., Ghorban, K., and Dadmanesh, M. 2020. Combined effects of *Allium sativum* and *Cuminum cyminum* essential oils on planktonic and biofilm forms of *Salmonella typhimurium* isolates. *3 Biotech* 10 (7):315. doi:10.1007/s13205-020-02286-2.
- Hammer, K. A., Carson, C. F., and Riley, T. V. 2008. Frequencies of resistance to *Melaleuca alternifolia* (tea tree) oil and rifampicin in *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Enterococcus faecalis*. *International Journal of Antimicrobial Agents* 32 (2):170-173. doi:10.1016/j.ijantimicag.2008.03.013.

- Hammer, K. A., Carson, C. F., and Riley, T. V. 2012. Effects of *Melaleuca alternifolia* (tea tree) essential oil and the major monoterpene component terpinen-4-ol on the development of single-and multistep antibiotic resistance and antimicrobial susceptibility. *Antimicrobial Agents and Chemotherapy* 56 (2):909-915. doi:10.1128/aac.05741-11.
- Händel, N., Schuurmans, J. M., Feng, Y., Brul, S., and Kuile, B. H. t. 2014. Interaction between mutations and regulation of gene expression during development of *de novo* antibiotic resistance. *Antimicrobial Agents and Chemotherapy* 58 (8):4371-4379. doi:10.1128/aac.02892-14.
- Harjai, K., Kumar, R., and Singh, S. 2010. Garlic blocks quorum sensing and attenuates the virulence of *Pseudomonas aeruginosa*. *Federation of European Microbiological Societies Immunology & Medical Microbiology* 58 (2):161-168. doi:10.1111/j.1574-695X.2009.00614.x.
- Helmy, Y. A., Taha-Abdelaziz, K., Hawwas, H. A. E., Ghosh, S., AlKafaas, S. S., Moawad, M. M. M., Saied, E. M., Kassem, II, and Mawad, A. M. M. 2023. Antimicrobial resistance and recent alternatives to antibiotics for the control of bacterial pathogens with an emphasis on foodborne pathogens. *Antibiotics (Basel)* 12 (2):274. doi:10.3390/antibiotics12020274.
- Hjort, K., Fermér, E., Tang, P.-C., and Andersson, D. I. 2022. Antibiotic minimal selective concentrations and fitness costs during biofilm and planktonic growth. *Mbio* 13 (3):e01447-22. doi:10.1128/mbio.01447-22.
- Hung, T. T., Ngan, L. T. M., Viet, H., Hoang, N. V. M., and Hieu, T. T. 2023. Chemical composition and anti-*Helicobacter pylori* activity of essential oil from fresh fruits of *Litsea cubeba* (Lour.) Pers. *Journal of Essential Oil Research* 35 (3):207-219. doi:10.1080/10412905.2023.2167876.
- Iseppi, R., Di Cerbo, A., Aloisi, P., Manelli, M., Pellesi, V., Provenzano, C., Camellini, S., Messi, P., and Sabia, C. 2020. *In vitro* activity of essential oils against planktonic and biofilm cells of extended-spectrum  $\beta$ -lactamase (ESBL)/carbapenamase-producing Gram-negative bacteria involved in human nosocomial infections. *Antibiotics (Basel)* 9 (5):272. doi:10.3390/antibiotics9050272.

- Iseppi, R., Mariani, M., Benvenuti, S., Truzzi, E., and Messi, P. 2023. Effects of *Melaleuca alternifolia* Chell (Tea Tree) and *Eucalyptus globulus* Labill. essential oils on antibiotic-resistant bacterial biofilms. *Molecules* 28 (4):1671. doi:10.3390/molecules28041671.
- Jafri, H., Ansari, F. A., and Ahmad, I. 2019. Prospects of essential oils in controlling pathogenic biofilm. In: Press, A. (ed.) *In New look to phytomedicine*. Elsevier 9:203-236. doi:10.1016/B978-0-12-814619-4.00009-4.
- Jafri, H., Husain, F. M., and Ahmad, I. 2014. Antibacterial and antibiofilm activity of some essential oils and compounds against clinical strains of *Staphylococcus aureus*. *Journal of Biomedical and Therapeutic Sciences* 1 (1):65-71. ISSN:2394-2274.
- Jaramillo-Colorado, B., Olivero-Verbel, J., Stashenko, E. E., Wagner-Döbler, I., and Kunze, B. 2012. Anti-quorum sensing activity of essential oils from Colombian plants. *Natural Product Research* 26 (12):1075-1086. doi:10.1080/14786419.2011.557376.
- Jiang, Q., Feng, M., Ye, C., and Yu, X. 2022. Effects and relevant mechanisms of non-antibiotic factors on the horizontal transfer of antibiotic resistance genes in water environments: A review. *Science of The Total Environment* 806:150568. doi:10.1016/j.scitotenv.2021.150568
- Jini, D. 2023. Biological Applications of Essential Oil. *Essential oils: Extraction methods and applications*:361-380. doi:10.1002/9781119829614.ch16
- Kafa, A. H. T., Aslan, R., Celik, C., and Hasbek, M. 2022. Antimicrobial synergism and antibiofilm activities of *Pelargonium graveolens*, *Rosemary officinalis*, and *Mentha piperita* essential oils against extreme drug-resistant *Acinetobacter baumannii* clinical isolates. *Zeitschrift für Naturforschung C* 77 (3-4):95-104. doi:10.1515/znc-2021-0079.
- Kaufmann, G. F., Park, J., and Janda, K. D. 2008. Bacterial quorum sensing: a new target for anti-infective immunotherapy. *Expert Opinion on Biological Therapy* 8 (6):719-724. doi:10.1517/14712598.8.6.719.
- Kerekes, E. B., Vidács, A., Takó, M., Petkovits, T., Vágvölgyi, C., Horváth, G., Balázs, V. L., and Krisch, J. 2019. Anti-biofilm effect of selected essential oils and main components on mono- and polymicrobial bacterial cultures. *Microorganisms* 7 (9):345. doi:10.3390/microorganisms7090345.

- Khameneh, B., Iranshahy, M., Soheili, V., and Fazly Bazzaz, B. S. 2019. Review on plant antimicrobials: a mechanistic viewpoint. *Antimicrobial Resistance & Infection Control* 8 (1):1-28. doi:10.1186/s13756-019-0559-6.
- Khan, M. S. A., Zahin, M., Hasan, S., Husain, F. M., and Ahmad, I. 2009. Inhibition of quorum sensing regulated bacterial functions by plant essential oils with special reference to clove oil. *Letters in applied microbiology* 49 (3):354-360. doi:10.1111/j.1472-765X.2009.02666.x.
- Khosakueng, M., Taweekhaisupapong, S., Boonyanugomol, W., Prapatpong, P., Wongkaewkhiaw, S., and Kanthawong, S. 2024. *Cymbopogon citratus* L. essential oil as a potential anti-biofilm agent active against antibiotic-resistant bacteria isolated from chronic rhinosinusitis patients. *Biofouling* 40 (1):26-39. doi:10.1080/08927014.2024.2305387.
- Kim, Y.-G., Lee, J.-H., Kim, S.-I., 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. doi:10.1016/j.ijfoodmicro.2014.11.028.
- Kim, Y.-G., Lee, J.-H., Gwon, G., Kim, S.-I., Park, J. G., and Lee, J. 2016. Essential oils and eugenols inhibit biofilm formation and the virulence of *Escherichia coli* O157:H7. *Scientific Reports* 6 (1):36377. doi:10.1038/srep36377.
- Kryvtsova, M., Fedkiv, O., Hrytsyna, M., and Salamon, I. 2020. Anti-microbial, and anti-biofilm-forming properties of *Origanum vulgare* L. essential oils on *Staphylococcus aureus* and its antioxidant action. *Studia Biologica* 14 (2):27-38. doi:10.30970/sbi.1402.621.
- Lahkimi, A., Bouchra, L., chaouch, m., Nechad, I., and Eloutassi, N. 2020. Antibacterial, antifungal and antioxidant activity of *Lavandula Angustifolia* of the middle atlas central (Morocco). *Moroccan Journal of Chemistry* 8 (4):905-918. ISSN:2351-812X.
- Lang, G., and Buchbauer, G. 2012. A review on recent research results (2008–2010) on essential oils as antimicrobials and antifungals. A review. *Flavour and Fragrance Journal* 27 (1):13-39. doi:10.1002/ffj.2082.
- Lee, K., Lee, J.-H., Kim, S.-I., Cho, M. H., and Lee, J. 2014. Anti-biofilm, anti-hemolysis, and anti-virulence activities of black pepper, cananga, myrrha oils, and nerolidol against

- Staphylococcus aureus*. *Applied Microbiology and Biotechnology* 98 (22):9447-9457. doi:10.1007/s00253-014-5903-4.
- Leigh-de Rapper, S., and van Vuuren, S. F. 2020. Odoriferous therapy: A review identifying essential oils against pathogens of the respiratory tract. *Chemistry & Biodiversity* 17 (6):e2000062. doi:10.1002/cbdv.202000062.
- Leyva-López, N., Gutiérrez-Grijalva, E. P., Vazquez-Olivo, G., and Heredia, J. B. 2017. Essential oils of oregano: Biological activity beyond their antimicrobial properties. *Molecules* 22 (6):989. doi:10.3390/molecules22060989.
- Li, A., Shi, C., Qian, S., Wang, Z., Zhao, S., Liu, Y., and Xue, Z. 2022. Evaluation of antibiotic combination of *Litsea cubeba* essential oil on *Vibrio parahaemolyticus* inhibition mechanism and anti-biofilm ability. *Microbial Pathogenesis* 168:105574. doi:10.1016/j.micpath.2022.105574.
- Li, Y.-H., and Tian, X. 2012. Quorum sensing and bacterial social interactions in biofilms. *Sensors* 12 (3):2519-2538. doi:10.3390/s120302519.
- Li, Z., Xie, J., Yang, J., Liu, S., Ding, Z., Hao, J., Ding, Y., Zeng, Z., and Liu, J. 2021. Pathogenic characteristics and risk factors for ESKAPE pathogens infection in burn patients. *Infection and Drug Resistance* 14:4727-4738. doi:10.2147/IDR.S338627.
- Liu, C., Cheng, F., Aisa, H. A., and Maiwulanjiang, M. 2023. Comprehensive study of components and antimicrobial properties of essential oil extracted from *Carum Carvi* l. seeds. *Antibiotics* 12 (3):591. doi:10.3390/antibiotics12030591.
- Ma, Y.-X., Wang, C.-Y., Li, Y.-Y., Li, J., Wan, Q.-Q., Chen, J.-H., Tay, F. R., and Niu, L.-N. 2020. Considerations and caveats in combating eskape pathogens against nosocomial infections. *Advanced Science* 7 (1):1901872. doi:10.1002/advs.201901872.
- Mahumane, G. D. 2016. Antimicrobial activity and chemical analysis of *Eucalyptus radiata* leaf essential oil. Master of Pharmacy, Department of Pharmacy and Pharmacology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa.
- Mansuri, A., Lokhande, K., Kore, S., Gaikwad, S., Nawani, N., Swamy, K. V., Junnarkar, M., and Pawar, S. 2022. Antioxidant, anti-quorum sensing, biofilm inhibitory activities and chemical composition of patchouli essential oil: *In vitro* and *in silico* approach. *Journal*

- Manyi-Loh, C., Mamphweli, S., Meyer, E., and Okoh, A. 2018. Antibiotic use in agriculture and its consequential resistance in environmental sources: Potential public health implications. *Molecules* 23 (4). doi:10.3390/molecules23040795.
- Massarine, N. C. M., de Souza, G. H. A., Nunes, I. B., Salomé, T. M., Barbosa, M. D. S., Faccin, I., Rossato, L., and Simionatto, S. 2023. How Did COVID-19 impact the antimicrobial consumption and bacterial resistance profiles in Brazil? *Antibiotics (Basel)* 12 (9):1374. doi:10.3390/antibiotics12091374.
- Masyita, A., Sari, R. M., Astuti, A. D., Yasir, B., Rumata, N. R., Emran, T. B., Nainu, F., and Simal-Gandara, J. 2022. Terpenes and terpenoids as main bioactive compounds of essential oils, their roles in human health and potential application as natural food preservatives. *Food chemistry: X* (13):100217. doi:10.1016/j.fochx.2022.100217.
- Melo, A. D. B., Amaral, A. F., Schaefer, G., Luciano, F. B., de Andrade, C., Costa, L. B., and Rostagno, M. H. 2015. Antimicrobial effect against different bacterial strains and bacterial adaptation to essential oils used as feed additives. *Canadian journal of veterinary research* 79 (4):285-289. ISSN:0830-9000.
- Merghni, A., Haddaji, N., Bouali, N., Alabbosh, K. F., Adnan, M., Snoussi, M., and Noumi, E. 2022. Comparative study of antibacterial, antibiofilm, antiswarming and antiquorum sensing activities of *Origanum vulgare* essential oil and terpinene-4-ol against pathogenic bacteria. *Life* 12 (10):1616. doi:10.3390/life12101616.
- Merghni, A., Marzouki, H., Hentati, H., Aouni, M., and Mastouri, M. 2016. Antibacterial and antibiofilm activities of *Laurus nobilis* L. essential oil against *Staphylococcus aureus* strains associated with oral infections. *Current Research in Translational Medicine* 64 (1):29-34. doi:10.1016/j.patbio.2015.10.003.
- Michaelis, C., and Grohmann, E. 2023. Horizontal gene transfer of antibiotic resistance genes in biofilms. *Antibiotics* 12 (2):328. doi:10.3390/antibiotics12020328.
- Millezi, A. F., Costa, K. A. D., Oliveira, J. M., Lopes, S. P., Pereira, M. O., and Piccoli, R. H. 2019. Antibacterial and anti-biofilm activity of cinnamon essential oil and eugenol. *Ciência Rural* 49:e20180314. doi:10.1590/0103-8478cr20180314.

- Mirzaei, R., Yousefimashouf, R., Arabestani, M. R., Sedighi, I., and Alikhani, M. Y. 2022. The issue beyond resistance: Methicillin-resistant *Staphylococcus epidermidis* biofilm formation is induced by subinhibitory concentrations of cloxacillin, cefazolin, and clindamycin. *PLoS One* 17 (11):e0277287. doi:10.1371/journal.pone.0277287
- 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. doi:10.3389/fmicb.2019.00539.
- Munita, J. M., and Arias, C. A. 2016. Mechanisms of antibiotic resistance. In: Kudva, I. T., Ames, I. A. and Zhang, Q. (eds.) *Virulence Mechanisms of Bacterial Pathogens* Chile: Microbiology Spectrum 4 (2):481-511. doi:10.1128/9781555819286.ch17.
- Mutuku, C., Gazdag, Z., and Melegh, S. 2022. Occurrence of antibiotics and bacterial resistance genes in wastewater: resistance mechanisms and antimicrobial resistance control approaches. *World Journal of Microbiology and Biotechnology* 38 (9):152. doi:10.1007/s11274-022-03334-0.
- Nazzaro, F., Fratianni, F., De Martino, L., Coppola, R., and De Feo, V. 2013a. Effect of essential oils on pathogenic bacteria. *Pharmaceuticals (Basel)* 6 (12):1451-74. doi:10.3390/ph6121451.
- Nazzaro, F., Polito, F., Amato, G., Caputo, L., Francolino, R., D'Acierno, A., Fratianni, F., Candido, V., Coppola, R., and De Feo, V. 2022. Chemical composition of essential oils of bulbs and aerial parts of two cultivars of *Allium Sativum* and their antibiofilm activity against food and nosocomial pathogens. *Antibiotics* 11 (6):724. doi:10.3390/antibiotics11060724.
- Negreiros, M. O., Pawlowski, Â., Zini, C. A., Soares, G. L. G., Motta, A. d. S., and Frazzon, A. P. G. 2016. Antimicrobial and antibiofilm activity of *Baccharis psiadioides* essential oil against antibiotic-resistant *Enterococcus faecalis* strains. *Pharmaceutical Biology* 54 (12):3272 - 3279. doi:10.1080/13880209.2016.1223700.
- Nelson, R. R. 2000. Selection of resistance to the essential oil of *Melaleuca alternifolia* in *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy* 45 (4):549-550. doi:10.1093/jac/45.4.549.

- Nenaah, G. E., Alasmari, S., Almadiy, A. A., Albogami, B. Z., Shower, D. M., and Fadl, A. E. 2023. Bio-efficacy of *Salvia officinalis* essential oil, nanoemulsion and monoterpene components as eco-friendly green insecticides for controlling the granary weevil. *Industrial Crops and Products* 204:117298. doi:10.1016/j.indcrop.2023.117298.
- Noumi, E., Merghni, A., M, M. A., Haddad, O., Akmadar, G., De Martino, L., Mastouri, M., Ceylan, O., Snoussi, M., Al-Sieni, A., and De Feo, V. 2018. *Chromobacterium violaceum* and *Pseudomonas aeruginosa* PAO1: Models for evaluating anti-quorum sensing activity of *Melaleuca alternifolia* essential oil and its main component terpinen-4-ol. *Molecules* 23 (10):2672. doi:10.3390/molecules23102672.
- Olivero-Verbel, J., Barreto-Maya, A., Bertel-Sevilla, A., and Stashenko, E. E. 2014. Composition, anti-quorum sensing and antimicrobial activity of essential oils from *Lippia alba*. *Brazilian Journal of Microbiology* 45:759-767. doi:10.1590/S1517-83822014000300001.
- Oppenheimer-Shaanan, Y., Steinberg, N., and Kolodkin-Gal, I. 2013. Small molecules are natural triggers for the disassembly of biofilms. *Trends in Microbiology* 21 (11):594-601. doi:10.1016/j.tim.2013.08.005.
- Orchard, A., Sandasi, M., Kamatou, G. P. P., Viljoen, A., and van Vuuren, S. 2017. The *in vitro* antimicrobial activity and chemometric modelling of 59 commercial essential oils against pathogens of dermatological relevance. *Chemistry and Biodiversity* 14 (1):e1600218. doi:10.1002/cbdv.201600218.
- Orchard, A., Van Vuuren, S., Viljoen, A., and Kamatou, G. 2018. The *in vitro* antimicrobial evaluation of commercial essential oils and their combinations against acne. *International journal of cosmetic science* 40 (3):226-243. doi:10.1111/ics.12456.
- Özfenerci, M., Bali, E. B., Erdönmez, D., and Çalışkan, U. K. 2022. Aromatherapeutic essential oils and their pharmaceutical combinations: Tools for inhibition of quorum sensing activity and biofilm formation of human pathogens. *İstanbul Journal of Pharmacy* 52 (1):54-63. doi:10.26650/IstanbulJPharm.2022.998774.
- Panda, S., Sahoo, S., Tripathy, K., Singh, Y. D., Sarma, M. K., Babu, P. J., and Singh, M. C. 2020. Essential oils and their pharmacotherapeutics applications in human diseases. *Advances in Traditional Medicine* 22:1-15. doi:10.1007/S13596-020-00477-z.

- Panda, S. K., Buroni, S., Swain, S. S., Bonacorsi, A., da Fonseca Amorim, E. A., Kulshrestha, M., da Silva, L. C. N., and Tiwari, V. 2022. Recent advances to combat ESKAPE pathogens with special reference to essential oils. *Frontiers in Microbiology* 13:1029098. doi:10.3389/fmicb.2022.1029098.
- Patterson, J. E., McElmeel, L., and Wiederhold, N. P. 2019. *In vitro* activity of essential oils against Gram-positive and Gram-negative clinical isolates, including carbapenem-resistant *Enterobacteriaceae*. *Open Forum Infectious Diseases* 6 (12):ofz502. doi:10.1093/ofid/ofz502.
- Pekmezovic, M., Aleksic, I., Barac, A., Arsic-Arsenijevic, V., Vasiljevic, B., Nikodinovic-Runic, J., and Senerovic, L. 2016. Prevention of polymicrobial biofilms composed of *Pseudomonas aeruginosa* and pathogenic fungi by essential oils from selected *Citrus* species. *Federation of European Microbiological Societies Pathogens and Disease* 74 (8):ftw102. doi:10.1093/femspd/ftw102.
- Piddock, L. J. 2012. The crisis of no new antibiotics—what is the way forward? *The Lancet infectious diseases* 12 (3):249-253. doi:10.1016/S1473-3099(11)70316-4.
- Piras, A., Gonçalves, M. J., Alves, J., Falconieri, D., Porcedda, S., Maxia, A., and Salgueiro, L. 2018. *Ocimum tenuiflorum* L. and *Ocimum basilicum* L., two spices of Lamiaceae family with bioactive essential oils. *Industrial Crops and Products* 113:89-97. doi:10.1016/j.indcrop.2018.01.024.
- Poli, J. P., Guinoiseau, E., de Rocca Serra, D., Sutour, S., Paoli, M., Tomi, F., Quilichini, Y., Berti, L., and Lorenzi, V. 2018. Anti-quorum sensing activity of 12 essential oils on *Chromobacterium violaceum* and specific action of *cis-cis-p*-menthenolide from *Corsican mentha suaveolens* ssp. *insularis*. *Molecules* 23 (9). doi:10.3390/molecules23092125.
- Prabuseenivasan, S., Jayakumar, M., and Ignacimuthu, S. 2006. *In vitro* antibacterial activity of some plant essential oils. *BMC Complementary and Alternative Medicine* 6 (1):39. doi:10.1186/1472-6882-6-39.
- Raal, A., Arak, E., and Orav, A. 2012. The content and composition of the essential oil found in *Carum carvi* L. commercial fruits obtained from different countries. *Journal of Essential Oil Research* 24 (1):53-59. doi:10.1080/10412905.2012.646016.

- Reichling, J. 2020. Anti-biofilm and virulence factor-reducing activities of essential oils and oil components as a possible option for bacterial infection control. *Planta Medica* 86 (08):520-537. doi:10.1055/a-1147-4671.
- Rodríguez-Lázaro, D., Alonso-Calleja, C., Oniciuc, E. A., Capita, R., Gallego, D., González-Machado, C., Wagner, M., Barbu, V., Eiros-Bouza, J. M., and Nicolau, A. I. 2018. Characterization of biofilms formed by foodborne methicillin-resistant *Staphylococcus aureus*. *Frontiers in Microbiology* 9:3004. doi:10.3389/fmicb.2018.03004.
- Roman, H., Niculescu, A.-G., Lazăr, V., and Mitache, M. M. 2023. Antibacterial efficiency of *Tanacetum vulgare* essential oil against ESKAPE pathogens and synergisms with antibiotics. *Antibiotics* 12 (11):1635. doi:10.3390/antibiotics12111635.
- 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. *Journal of Intercultural Ethnopharmacology* 5 (3):212-8. doi:10.5455/jice.20160331064446.
- Sanchez-Cid, C., Guironnet, A., Keuschnig, C., Wiest, L., Vulliet, E., and Vogel, T. M. 2022. Gentamicin at sub-inhibitory concentrations selects for antibiotic resistance in the environment. *International Society for Microbial Ecology Communications* 2 (1):29. doi:10.1038/s43705-022-00101-y.
- Sartorius, B., Gray, A. P., Weaver, N. D., Aguilar, G. R., Swetschinski, L. R., Ikuta, K. S., Mestrovic, T., Chung, E., Wool, E. E., and Han, C. 2024. The burden of bacterial antimicrobial resistance in the WHO African region in 2019: a cross-country systematic analysis. *The Lancet Global Health* 12 (2):e201-e216. doi:10.1016/S2214-109X(23)00539-9.
- Schelz, Z., Hohmann, J., Molnár, J. 2010. Recent advances in research of antimicrobial effects of essential oils and plant derived compounds on bacteria. In: Chattopadhyay, D. (ed.) *Ethnomedicine: A source of complementary therapeutics* Kerala, India: Research Signpost 37 (6):179-201. ISBN:978-81-308-0390-6.
- Sengupta, S., Chattopadhyay, M. K., and Grossart, H.-P. 2013. The multifaceted roles of antibiotics and antibiotic resistance in nature. *Frontiers in Microbiology* 4:47. doi:10.3389/fmicb.2013.00047.

- Serwecińska, L. 2020. Antimicrobials and antibiotic-resistant bacteria: A risk to the environment and to public health. *Water* 12 (12):3313. doi:10.3390/w12123313.
- Shin, H.-J., Yang, S., and Lim, Y. 2021. Antibiotic susceptibility of *Staphylococcus aureus* with different degrees of biofilm formation. *Journal of Analytical Science and Technology* 12 (1):1-7. doi:10.1186/s40543-021-00294-2.
- Singh, D., and Agarwal, V. 2021. Screening of antimicrobial, anti-quorum sensing activity and cytotoxicity of origanum oil against Gram-positive and Gram-negative bacteria. *Biomedicine* 41 (3):599-603. doi:10.51248/v41i3.1200.
- Singh, L. 2022. Essential oil-loaded polymeric film for antimicrobial application to a urinary catheter. Master of Pharmacy, Department of Pharmacy and Pharmacology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa.
- Sionov, R. V., and Steinberg, D. 2022. Targeting the holy triangle of quorum sensing, biofilm formation, and antibiotic resistance in pathogenic bacteria. *Microorganisms* 10 (6):1239. doi:10.3390/microorganisms10061239.
- Sokolović, D., Drakul, D., Joksimović, B., Lalović, N., Avram, N., Milić, M., Nogo-Živanović, D., and Mijović, B. 2022. Consumption of antibiotics in primary care setting before and during Covid-19 pandemic in Republic of Srpska, Bosnia and Herzegovina. *Antibiotics* 11 (10):1319. doi:10.3390/antibiotics11101319.
- Speck, S., Wenke, C., Feßler, A. T., Kacza, J., Geber, F., Scholtzek, A. D., Hanke, D., Eichhorn, I., Schwarz, S., Rosolowski, M., and Truyen, U. 2020. Borderline resistance to oxacillin in *Staphylococcus aureus* after treatment with sub-lethal sodium hypochlorite concentrations. *Heliyon* 6 (6):e04070. doi:10.1016/j.heliyon.2020.e04070.
- Spellberg, B., and Gilbert, D. N. 2014. The future of antibiotics and resistance: a tribute to a career of leadership by John Bartlett. *Clinical infectious diseases* 59 (suppl\_2):S71-S75. doi:10.1093/cid/ciu392.
- Stenger, L. D., Libardoni, G., Wagner Júnior, A., Zanela, J., Alves, L. T., Varpechoski, G. O., Lozano, E. R., and Potrich, M. 2021. Essential oils in pathogen resistance induction of *Eucalyptus benthamii* Maiden et Cabbage. *Ciência Rural* 51:e20190915. doi:10.1590/0103-8478cr20190915.

- Stennett, H. L., Back, C. R., and Race, P. R. 2022. Derivation of a precise and consistent timeline for antibiotic development. *Antibiotics (Basel)* 11 (9):1237. doi:10.3390/antibiotics11091237.
- Streit, F., Perl, T., Schulze, M. H., and Binder, L. 2016. Personalised beta-lactam therapy: basic principles and practical approach. *Journal of Laboratory Medicine* 40 (6):385-397. doi:10.1515/labmed-2016-0050.
- Szabó, M. A., Varga, G. Z., Hohmann, J., Schelz, Z., Szegedi, E., Amaral, L., and Molnár, J. 2010. Inhibition of quorum-sensing signals by essential oils. *Phytotherapy Research* 24 (5):782-6. doi:10.1002/ptr.3010.
- Taga, M. E., and Bassler, B. L. 2003. Chemical communication among bacteria. *Proceedings of the National Academy of Sciences* 100 (2):14549-14554. doi:10.1073/pnas.193451410.
- Taleb, M. H., Elmanama, A. A., Taleb, A. H., and Tawfick, M. M. 2023. Pre- and post-COVID-19 antimicrobial resistance profile of bacterial pathogens, a comparative study in a tertiary hospital. *The Journal of Infection in Developing Countries* 17 (5):597-609. doi:10.3855/jidc.17791. doi:10.3390/antibiotics12101539.
- Tapia-Rodriguez, M. R., Cantu-Soto, E. U., Vazquez-Armenta, F. J., Bernal-Mercado, A. T., and Ayala-Zavala, J. F. 2023. Inhibition of *Acinetobacter baumannii* biofilm formation by terpenes from oregano (*Lippia graveolens*) essential oil. *Antibiotics* 12 (10):1539.
- Tariq, S., Wani, S., Rasool, W., Bhat, M. A., Prabhakar, A., Shalla, A. H., and Rather, M. A. 2019. A comprehensive review of the antibacterial, antifungal and antiviral potential of essential oils and their chemical constituents against drug-resistant microbial pathogens. *Microbial pathogenesis* 134:103580. doi:10.1016/j.micpath.2019.103580.
- Tello, A., Austin, B., and Telfer, T. C. 2012. Selective pressure of antibiotic pollution on bacteria of importance to public health. *Environmental Health Perspectives* 120 (8):1100-6. doi:10.1289/ehp.1104650.
- Tsao, S.-m., and Yin, M.-c. 2001. *In vitro* activity of garlic oil and four diallyl sulphides against antibiotic-resistant *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. *Journal of Antimicrobial Chemotherapy* 47 (5):665-670. doi:10.1093/jac/47.5.665.

- Valdivieso-Ugarte, M., Gomez-Llorente, C., Plaza-Díaz, J., and Gil, Á. 2019. Antimicrobial, antioxidant, and immunomodulatory properties of essential oils: A Systematic review. *Nutrients* 11 (11):2786. doi:10.3390/nu11112786.
- Vázquez-Sánchez, D., Cabo, M. L., and Rodríguez-Herrera, J. J. 2015. Antimicrobial activity of essential oils against *Staphylococcus aureus* biofilms. *Food Science and Technology International* 21 (8):559-570. doi:10.1177/1082013214553996.
- Ventola, C. L. 2015. The antibiotic resistance crisis: part 1: causes and threats. *Pharmacy and therapeutics* 40 (4):277-83. ISSN:1052-1372.
- Wang, W., Li, D., Huang, X., Yang, H., Qiu, Z., Zou, L., Liang, Q., Shi, Y., Wu, Y., Wu, S., Yang, C., and Li, Y. 2019. Study on antibacterial and quorum-sensing inhibition activities of *Cinnamomum camphora* leaf essential oil. *Molecules* 24 (20):3792. doi:10.3390/molecules24203792.
- WHO. 2023. "Vaccines could avert half a million deaths associated with anti-microbial resistance a year." <https://www.who.int/news/item/28-07-2023-vaccines-could-avert-half-a-million-deaths-associated-with-anti-microbial-resistance-a-year#:~:text=A%20new%20study%20published%20in,vaccines%20to%20tackle%20priority%20pathogens>.
- Wu, X., Wang, H., Xiong, J., Yang, G.-X., Hu, J.-F., Zhu, Q., and Chen, Z. 2024. *Staphylococcus aureus* biofilm: Formulation, regulatory, and emerging natural products-derived therapeutics. *Biofilm* 7:100175. doi:10.1016/j.bioflm.2023.100175.
- Yang, Y., Hao, K., Jiang, M., Memon, F. U., Guo, L., Zhang, G., Liu, T., Wu, X., and Si, H. 2021. Transcriptomic analysis of drug-resistance *Acinetobacter baumannii* under the stress condition caused by *Litsea cubeba* L. essential oil via RNA sequencing. *Genes* 12 (7):1003. doi:10.3390/genes12071003.
- 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* 8:6. doi:10.2174/1874285801408010006.
- Yu, Z., Tang, J., Khare, T., and Kumar, V. 2020. The alarming antimicrobial resistance in ESKAPEE pathogens: Can essential oils come to the rescue? *Fitoterapia* 140:104433. doi:10.1016/j.fitote.2019.104433.

- Zhan, X., Tan, Y.-J., Lv, Y., Fang, J., Zhou, Y. M., Gao, X., Zhu, H., and Shi, C. 2022. The antimicrobial and antibiofilm activity of oregano essential oil against *Enterococcus faecalis* and its application in chicken breast. *Foods* 11 (15):2296. doi:10.3390/foods11152296.
- Zhao, X., Yu, Z., and Ding, T. 2020. Quorum-sensing regulation of antimicrobial resistance in bacteria. *Microorganisms* 8 (3):425. doi:10.3390/microorganisms8030425.

## APPENDIX A1 - Abstract for presentation (STHS)

---

---

### ARE ESSENTIAL OILS A SOLUTION TO COMBAT ANTIMICROBIAL RESISTANCE?

Keruné Naidoo, Sandy van Vuuren and Ané Orchard.

Department of Pharmacy and Pharmacology, University of the Witwatersrand.

It is well known that antimicrobial resistance is a global issue. Essential oils (EOs) are often studied as alternative antimicrobials due to their ability to exert noteworthy antimicrobial activity against a broad range of micro-organisms, including antimicrobial resistant ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) pathogens. It is assumed that EOs can withstand the development of resistance due to them being multi-component structures, however, this assumption lacks scientific validation. This study aimed to firstly investigate the antimicrobial activity of a selection of EOs and thereafter investigate the resistance development against five EOs in comparison to three antibiotics, namely, ciprofloxacin, erythromycin, and gentamicin. Anti-quorum sensing assays and Biofilm inhibition assays were performed to investigate resistance mechanisms. *Origanum vulgare* and *Thymus vulgaris* showed noteworthy results for the antimicrobial ( $\leq 1.00$  mg/mL) and anti-quorum sensing studies ( $> 70.00\%$  inhibition). Only *O. vulgare* exhibited significant biofilm inhibition ( $> 70.00\%$  inhibition). After repeated exposure to the EO at sub-inhibitory concentrations, a pattern of heteroresistance to antibiotics was observed in *Staphylococcus aureus* ATCC 6538. *Staphylococcus aureus* exposed to *Matricaria recutita*, *O. vulgare*, and *T. vulgaris* individually, showed an increase in the minimum inhibitory concentration (MIC) of ciprofloxacin, erythromycin, and gentamicin first at passage six and then increasing respectively. The MIC of the EOs remained consistent over the passages. These results show that, despite continuous exposure to the EOs, resistance to the EOs has not been identified, making it a very promising solution to the antimicrobial resistance crisis.

## APPENDIX A2 - Abstract for presentation (SAAB)

---

---

### ARE ESSENTIAL OILS A SOLUTION TO COMBAT ANTIMICROBIAL RESISTANCE?

Keruné Naidoo, Sandy van Vuuren and Ané Orchard.

Department of Pharmacy and Pharmacology, University of the Witwatersrand.

Antimicrobial resistance is a global concern, and essential oils (EOs) are frequently studied as potential alternative antimicrobials due to their ability to exhibit significant antimicrobial activity against a wide range of microorganisms, including ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) that are resistant to antimicrobials. While it's believed that EOs may resist resistance development due to their complex structures, this assumption lacks scientific confirmation. This study aimed to investigate the antimicrobial properties of selected EOs and their resistance development compared to three antibiotics (ciprofloxacin, erythromycin, and gentamicin). Anti-quorum sensing and biofilm inhibition assays were conducted to explore resistance mechanisms. *Origanum vulgare* and *Thymus vulgaris* showed significant antimicrobial activity ( $\leq 1.00$  mg/mL) and anti-quorum sensing effects ( $> 70.00\%$  inhibition). Only *O. vulgare* displayed significant biofilm inhibition ( $> 70.00\%$  inhibition). *Staphylococcus aureus* ATCC 6538 exhibited a pattern of heteroresistance to antibiotics after repeated exposure to sub-inhibitory concentrations of EOs, while resistance to the EOs themselves was not observed, indicating their potential as effective solutions to combat antimicrobial resistance.

## APPENDIX A3 - Abstract for presentation (MBRT)

---

---

### The Antimicrobial Potential of Essential Oils against the ESKAPE Pathogens

Keruné Naidoo<sup>1</sup>, Sandy van Vuuren<sup>1</sup>, Ané Orchard<sup>1</sup>

<sup>1</sup>University of the Witwatersrand, Faculty of Health Sciences, Department of Pharmacy and Pharmacology, 7 York Road, Parktown 2193, South Africa

It is widely known that antimicrobial resistance is a global phenomenon. Essential oils (EOs) are volatile and multi-component natural products. There is a vast number of studies investigating EOs as a potential substitute for antimicrobials due to their ability to effectively inhibit pathogen growth, including those that are resistant to antimicrobials, such as the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species). It is theorized that EOs can resist the development of resistance based on their multi-component structures, however, studies to validate this are limited. Thus, this study aimed to investigate the antimicrobial activity of 23 EOs and subsequently investigate resistance development to a selection based on antimicrobial activity of the EOs in comparison to ciprofloxacin, erythromycin, and gentamicin. Anti-quorum sensing assays and biofilm inhibition assays were conducted to explore the mechanisms of resistance. Notable results were observed for the antimicrobial (1.00 mg/mL), anti-quorum sensing (> 70.00% inhibition), and biofilm inhibition studies (> 70.00% inhibition) with *Origanum vulgare*. *Staphylococcus aureus* ATCC 6538 displayed a pattern of heteroresistance to antibiotics after repeated exposure to the EO at sub-inhibitory concentrations. *Staphylococcus aureus* exposed to *Matricaria recutita*, *Origanum vulgare*, and *Thymus vulgaris* exhibited an increase in the minimum inhibitory concentration (MIC) of ciprofloxacin, erythromycin, and gentamicin, first at passage six and more frequently against *S. aureus* exposed to *T. vulgaris*. The MIC of the EOs remained consistent throughout the passages. These findings indicate that, despite continuous exposure to the EOs, resistance to them has not been observed, thus presenting a highly promising solution to the antimicrobial crisis.

# APPENDIX B - Ethics waiver



## Human Research Ethics Committee (Medical)

Research Office Secretariat:  
Faculty of Health Sciences, Phillip Tobias Health Sciences Building, 3<sup>rd</sup> Floor, Office 301/2/4, 29 Princess of Wales Terrace, Parktown, 2193  
Private Bag 3, Wits 2050  
Office email: [HREC-Medical.ResearchOffice@wits.ac.za](mailto:HREC-Medical.ResearchOffice@wits.ac.za)  
Website: <https://www.wits.ac.za/research/researcher-support/research-ethics/ethics-committees/>

Ref: W-CBP-220504-02

04/05/2022

### TO WHOM IT MAY CONCERN:

**Waiver:** This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical)

**Investigator:** Miss Keruné Naidoo

**Supervisors:** Dr Ané Orchard and Prof Sandy van Vuuren

**Department:** Pharmacy and Pharmacology

**Project title:** Are essential oils a solution to combat antimicrobial resistance?

**Reason:** *In vitro* laboratory study. No human participants will be involved in the study.

**Dr CB Penny**

Chairperson: Human Research Ethics Committee (Medical)

Copy – HREC (Medical) Secretariat: Ms Zanele Ndlovu, Ms Mapula Ramaila and Mr Rhulani Mkansi

## APPENDIX C - Plagiarism declaration

---



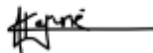
### PLAGIARISM DECLARATION TO BE SIGNED BY ALL HIGHER DEGREE STUDENTS

SENATE PLAGIARISM POLICY: APPENDIX ONE

I Keruné Naidoo (Student number: 1598125) am a student registered for the degree of Master of Pharmacy in the academic year 2024.

I hereby declare the following:

- I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.
- I confirm that the work submitted for assessment for the above degree is my own unaided work except where I have explicitly indicated otherwise.
- I have followed the required conventions in referencing the thoughts and ideas of others.
- I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.
- I have included as an appendix a report from "Turnitin" (or other approved plagiarism detection) software indicating the level of plagiarism in my research document.

Signature: 

Date: 28 March 2024

## APPENDIX D - Turnitin report

Fina MPharm dissertation KNaidoo 2024

ORIGINALITY REPORT

|                                |                               |                            |                             |
|--------------------------------|-------------------------------|----------------------------|-----------------------------|
| <b>15%</b><br>SIMILARITY INDEX | <b>9%</b><br>INTERNET SOURCES | <b>13%</b><br>PUBLICATIONS | <b>5%</b><br>STUDENT PAPERS |
|--------------------------------|-------------------------------|----------------------------|-----------------------------|

PRIMARY SOURCES

|          |   |               |
|----------|---|---------------|
| <b>1</b> | <b>www.mdpi.com</b><br>Internet Source  | <b>1%</b>     |
| <b>2</b> | Ané Orchard, Maxleene Sandasi, Guy Kamatou, Alvaro Viljoen, Sandy van Vuuren. " The Antimicrobial Activity and Chemometric Modelling of 59 Commercial Essential Oils against Pathogens of Dermatological Relevance ", Chemistry & Biodiversity, 2017<br>Publication | <b>1%</b>     |
| <b>3</b> | Submitted to University of Witwatersrand<br>Student Paper   | <b>1%</b>     |
| <b>4</b> | Submitted to University of Mauritius<br>Student Paper   | <b>&lt;1%</b> |
| <b>5</b> | "Posters", Clinical Microbiology and Infection, 4/2007<br>Publication   | <b>&lt;1%</b> |
| <b>6</b> | R.A. Khan, S.F. van Vuuren. " Essential oil combinations against and - the causative agents of gas gangrene ", Journal of Applied Microbiology, 2021<br>Publication   | <b>&lt;1%</b> |

