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Anti-virulence activities of selected culinary herbs against *Pseudomonas aeruginosa*

by

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DECLARATION

I, Onana Nguini Alphonse Honore, declare that the thesis/dissertation, which I hereby submit for the degree of Master of Science (MSc) in Microbiology at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature: 

Date: 2021/11/05

DEDICATION

I dedicate this thesis to my father, the late Onana Daniel, you always thought me to behave like a responsible and respectable man. My late uncle, Atangana, always welcoming me with so much joy every time I reached home, as well my late friend that I always considered as my own brother, Yesse Diramba. I wish you could have stayed longer to witness the end of my MSc journey. You are not forgotten; this is for you.

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ABSTRACT

Pseudomonas aeruginosa is a Gram-negative opportunistic bacterium that causes severe infections such as cystic fibrosis (CF), skin infections and urinary tract infections (UTI), especially in immunocompromised patients. The pathogen relies on its virulence factors that get activated following a successful entry inside the host, resulting in diseases causing infections. Conventional techniques to battle this pathogen include the use of antibiotics. However, these antibiotics have proven to cause an increased resistance of the pathogen, which drives the need to come up with other means of fighting emerging multi-resistant pathogenic strains of *P. aeruginosa*. Alternatively, microbial pathogenicity can be attenuated by disturbing the communication system between bacteria, known as quorum sensing (QS). Therefore, to manage these bacterial infections, a novel strategy of anti-quorum sensing (AQS) could be used, as it exerts no selective pressure on bacterial cells, but only disrupts the bacterial communication. This study aimed to determine antivirulence properties of selected culinary herbs against *Pseudomonas aeruginosa.*

The seven herbs, *Apium graveolens*, *Coriandrum sativum*, *Rosmarinus officinalis*, *Salvia africana lutea*, *Salvia officinalis*, *Syzygium aromaticum* and *Thymus vulgaris* were selected based on their reported health benefits they possess against diseases and ailments. The seven herbs were extracted with solvents of different polarities (water, hexane, ethyl acetate, methanol and dichloromethane), thereafter and determining minimum inhibitory concentrations (MIC) by microdilution assay against *Pseudomonas aeruginosa*. The *Chromobacterium violaceum* (biomonitor strain), which produces the purple violacein, was used for qualitative and quantitative AQS activities using the active extracts. The antivirulence assays (biofilm formation, elastase and pyocyanin productions, and twitching, swimming and swarming motility) of active extracts were evaluated against *P. aeruginosa*. Additionally, a chemical profiling of the active extracts was performed using gas chromatography coupled with mass spectrometry (GCMS) to identify possible compounds potentially responsible for the desired activities.

Eleven extracts of *Salvia officinalis* (hexane and DCM), *Rosmarinus officinalis* (hexane and aqueous), *Syzygium aromaticum* (ethyl acetate, methanol, and DCM), *Coriandrum sativum* (ethyl acetate, methanol, hexane and DCM) displayed noteworthy MIC values of 1.00 mg/ml against *P. aeruginosa.* Moreover, the qualitative AQS activity revealed only two hexane extracts of *Syzygium aromaticum* and *Coriandrum sativum* potentially possess some AQS potential with inhibition zones of 9.00 mm and 16.00 mm at sub-inhibitory MIC (0.35 mg/ml), respectively. Quantitatively, *C. sativum* (hexane) extract showed higher percentage inhibition of 69% while and *S. aromaticum* (hexane) showed least AQS activity of 24% at 0.50 mg/ml. Only AQS active hexane extracts of *S. aromaticum* and *C. sativum* were subjected testing against virulence factors of *P. aeruginosa*. The two extracts of *C. sativum* and *S. aromaticum* (hexane) demonstrated percentage high biofilm inhibitions of 70.3% and 80.1% at 1.00 mg/ml, respectively. Furthermore, *P. aeruginosa* showed susceptibility with reduced twitching motility when treatment with 1.00 mg/ml of the extracts *C. sativum* hexane (36%) and *S. aromaticum* (43%). *C. sativum* hexane extract also reduced the swarming motility of *Pseudomonas aeruginosa* by 35% at 1.00 mg/ml. Whereas the hexane extract of *S. aromaticum* displayed 59% inhibition for elastase production. From the hexane extracts of *S. aromaticum* and *C. sativum*, 27 compounds were identified using GC-MS. Of the 27 compounds belonging to different classes of chemicals, some are known to possess a potential in inhibiting *Pseudomonas* sp at the quorum sensing level, such as Tetradecanoic acid, linoleic acid as well as phytol.

This study validated and highlighted the herbs of S. *aromaticum,* and *C. sativum* possess antibacterial activities and antivirulence properties that could be used in attenuation of resistant *P. aeruginosa*, thereby used in management of its infections.

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CHAPTER 1 - INTRODUCTION

1.1. Background

The discovery of antibiotics was once a remarkable event in medical history due to the countless lives it saved from infectious diseases that used to be fatal to humans. Despite their benefits in reducing infections, antibiotics have become inefficient over the years (Shallcross *et al.*, 2015). This is due to the rise of multidrug-resistant (MDR) pathogenic strains, caused by the overuse and misuses of antibiotics, creating selection pressures (Ventola, 2015). In South Africa, the Department of Health (NDoH.) announced that antibiotics consumption was 74% in 2015, this information raises some concerns regarding the emergence of new MDR strains (National Department of Health, 2018)

Pseudomonas aeruginosa is well-known to cause diseases and the leading cause of high mortality rates in patients with immunocompromised systems, such as patients with the human immunodeficiency virus (HIV), as well as patients with cystic fibrosis (CF) and cancers (Karatuna & Yagci, 2010). The pathogen causes several symptoms that are organ-related but the infection wounds generally display a greenish exudate specific to this pathogen (Dos Santos Moraes *et al.*, 2008). *Pseudomonas aeruginosa* is viewed as a serious threat by the World Health Organization (WHO) and therefore, requires critical attention in research when it comes to finding new ways to combat the pathogen and its related infections. In South Africa, this pathogen is being detected in private hospitals as well (Adjei *et al.*, 2018). The pathogens are difficult to eradicate due to their ability to gain resistance to antibiotics via various mechanisms such as the modification of the antibiotic binding site, the degradation of the antibiotic, the efflux of the antibiotic via transporters and finally, the decrease in membrane permeability to prevent the uptake of the antibiotic (Munita & Arias, 2016). This resistance allows for the survival of the pathogen, which can consequently cause disease.

To cause diseases in patients, *Pseudomonas aeruginosa* relies massively on its virulence factors (Rutherford & Bassler, 2012). These virulence factors only activate once the pathogen is inside a suitable host and are mediated by a process known as quorum sensing (QS) (Niu *et al.*, 2006). Quorum sensing allows for the cell-to-cell communication of the pathogen, via secretion and detection of signaling molecules, resulting in the activation of virulence genes such as those that encode for toxins secretion, biofilms formation and motility (Niu *et al.*, 2006). These virulence genes are regulated by specific quorum-sensing systems (QSS) in

Pseudomonas aeruginosa: *las, pqs, iqs* and *rhl* (Lee & Zhang, 2015). Among the important virulence factors in *Pseudomonas aeruginosa*, elastase is one example. It is a protease encoded by the *las* system involved in QS (Spencer *et al.*, 2010). This protease facilitates the invasion of *Pseudomonas aeruginosa* by breaking down the host elastin tissues. This can result in urinary tract infections (UTI) (Spencer *et al.*, 2010). Additionally, like other microorganisms, *Pseudomonas aeruginosa* needs to utilize iron in the surrounding environment to complete its metabolic processes. This pathogen can switch its iron uptake by making use of siderophores such as pyoverdine, another virulence factor that binds iron molecules (Cornelis & Dingemans, 2013). This virulence factor is important to promote the survival of the pathogen in harsh, low-iron content environments such as the bladder. This also usually results in additional UTIs (Cornelis & Dingemans, 2013). Furthermore, the pathogen makes use of another important virulence factor to cause infections, this one is known as pyocyanin. Pyocyanin is a blue redox molecule involved in the production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-), two compounds that are toxic to the host cells resulting in cell death (Hall *et al.*, 2016). Pyocyanin also causes the release of extracellular DNA, a component involved in biofilm formation, an important virulence mechanism (Das & Manefield, 2012, Rasamiravaka *et al.*, 2015).

Biofilms are structures formed by communities of bacteria that are adhering to a surface and are surrounded by a protective exopolymeric substance (EPS) (Verderosa *et al.*, 2019). The EPS layer promotes the survival and persistence of the pathogen by providing a protective layer that prevents antibiotics from reaching the bacterial cells (Verderosa *et al.*, 2019). Additionally, the EPS maintains the structure of the biofilm, captures nutrients in the environment, shields the pathogen from the immune system and keep the pathogenic bacterial cells close to one another, enhancing bacterial cell-to-cell communication or quorum sensing even more (Flemming *et al.*, 2007, Verderosa *et al.*, 2019).

Propitiously, vast information is known about the QS process, thus creating an opportunity to target *Pseudomonas aeruginosa* infections differently to the antibiotic mechanism. Interfering with the signaling molecules has become a very appealing solution. This disruption in bacterial communications results in attenuated virulence rather than the direct killing of the pathogens (Chong *et al.*, 2011).

Interfering with QS is known as antiquorum sensing (AQS). The first AQS compound was discovered by Rasmussen *et al.* (2000) in *Delisea pulchra*, an alga. This compound was a halogenated furanone that was effective for inhibiting the swarming motility of *Serratia liquefaciens* MG1. This discovery highlighted the potential for fighting bacterial infections with

AQS compounds instead of antibiotics. It is highly likely that algae are not the only source of AQS compounds. This forces the shift to research other possible sources of AQS compounds. Considering that some culinary herbs have proven antibacterial properties, the possibility of finding potential AQS compounds cannot be omitted.

Herbs are products that are used daily for culinary purposes. A classic example includes *Ocimum basilicum* (basil), with proven antimicrobial properties (Kaya *et al.*, 2008). In terms of AQS research, Li *et al.* (2018) provided more evidence on the AQS properties of *Allium sativum,* in which they found that the compound Diallyl Disulphide (DADS) inhibited the elastase and pyocyanin productions, the biofilms formation as well as the swarming motility in *Pseudomonas aeruginosa*. On the other hand, Walker *et al.* (2004), and Viswanathan *et al.* (2015) demonstrated that the AQS properties of *Ocimum basilicum* were due to the presence of Rosmarinic Acid (RA) that was able to reduce the biofilm formation in *Pseudomonas aeruginosa*. These findings reinforced the vision that indeed, culinary herbs, can be used as sources of novel AQS compounds.

1.2. Motivation of the study

Pseudomonas aeruginosa is responsible for major nosocomial infections, yet resistant to numerous antibiotics such as β-lactams, aminoglycosides and fluoroquinolones (Adjei *et al.*, 2018). Additionally, *Pseudomonas aeruginosa* has a 75% high mortality rate, particularly in adult patients with underlying conditions (Perovic *et al.*, 2008). Most treatments still rely on the use of antibiotics; however, the use of these antibiotics is an accelerating factor in the emergence of MDR strains. This emboldens and drives the search to identify and adopt new methods of fighting infections against MDR strains. Targeting QS appears as a plausible approach to overcome the antibiotic resistance problem. However, this requires the identification of various sources to find compounds with the potential to interfere with QS.

Plants are reported as a repertoire of novel compounds (Alagarasan *et al.*, 2017). Culinary herbs fit in the category of novel compound sources and have been used extensively in human history primarily for culinary purposes and also medicinal purposes (Jiang, 2019). This study will focus on the AQS properties of selected culinary herbs against *Pseudomonas aeruginosa*.

1.3. Problem statement

Pseudomonas aeruginosa is a major pathogen. Because of its resistance to antibiotics, most antibiotic therapies are ineffective, hence alternative approaches or treatments to combat *P. aeruginosa* and its infections are imperative. This situation forces researchers to find such new ways to overcome this problem by finding potential AQS from natural products both *in vitro* and *in silico*.

1.4. Hypothesis

Selected South African (SA) culinary herbs contain compounds that can hinder the QS of *Pseudomonas aeruginosa*, rendering the pathogen less virulent.

1.5. Aim

This study aimed to study the antivirulence properties of the selected culinary herbs against *Pseudomonas aeruginosa* virulence factors.

1.6. Objectives

After selecting seven herbs and subjecting them to extraction with solvents of different polarities to recover plant compounds, the following objectives were followed for the successful execution of this study.

- To validate the antibacterial activities by determining the minimum inhibitory concentrations (MICs) of the plant extracts against *Pseudomonas aeruginosa*.
- To perform AQS activities by qualitative and quantitative measurements of violacein inhibition by the plant extracts, against *Chromobacterium violaceum*.
- To assess the effect of the plants extracts on *P. aeruginosa* virulence factors (biofilms inhibition, twitching motility, swarming motility, swimming motility, elastase inhibition, pyocyanin inhibition).
- To perform a chemical profiling of the active extracts using gas-chromatography coupled with mass spectrometry (GCMS)

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CHAPTER 2 - LITERATURE REVIEW

2. Overview

This review addresses one specific MDR bacterium, *Pseudomonas aeruginosa*, its importance as well as its complications in patients, and how it acquires resistance. Furthermore, it highlights how this pathogen manages its virulence factors via QS. Lastly, the strategy that can be employed to disrupt this QS as well as briefly discussing a select group of herbs that can potentially serve the purpose of interfering with the QS in *P. aeruginosa* are reviewed.

2.1. *Pseudomonas aeruginosa* and resistance

Once *Pseudomonas aeruginosa* establishes itself inside a host, the membrane can become impermeable to antibiotics preventing the penetration of drugs inside the cells. This property is granted by the presence of a lipopolysaccharide (LPS) layer present in the cell wall (Bassetti *et al.*, 2018). The pathogen also makes use of efflux pumps to actively pump out specific drugs prior to reaching their targets. Some efflux pumps have been identified and known to actively transport a wide range of drugs outside the cell. These efflux pumps are MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM and transport out a wide range of antibiotics such as quinolones, tetracyclines, carbapenems and many more (Bassetti *et al.*, 2018).

Pseudomonas aeruginosa can also express β-lactamases in its periplasmic space. These lactamase enzymes, encoded by *ampC,* are capable of cleaving β-lactam rings present in certain antibiotics such as penicillin, carbapenems or cephalosporins, consequently inactivating them (Tooke *et al.*, 2019). The production of these enzymes is strongly induced by carbapenems.

In addition, resistance can also occur through exposure to sub-inhibitory concentrations of antibacterial drugs. This type of resistance does not require mutational changes but is rather adaptive (Bassetti *et al.*, 2018, Pachori *et al.*, 2019). As example, polymyxin B targets the LPS of *Pseudomonas aeruginosa.* To prevent the binding of polymyxin B, *Pseudomonas*

aeruginosa alters its lipid A in the LPS, by adding phosphoethanolamine (PEtN) and 4-amino-4-deoxy-L-Arabinose (L-Ara4N) (Bassetti *et al.*, 2018). This alteration reduces the negative charge on the LPS, consequently affecting the binding of the Polymyxin B (Olaitan *et al.*, 2014, Bassetti *et al.*, 2018).

Administering multiple drugs to eradicate multi-resistant *Pseudomonas* pathogens is a daunting task as the bacterium possesses several mechanisms of resistance that allows it to survive and adapt to the presence of drugs when needed. The pathogen is highly versatile.

2.2. *Pseudomonas aeruginosa* pathogenesis

Infections from this pathogen can result in bacteraemia, which is the direct presence of bacterial cells in the bloodstream (Tam *et al.*, 2010). Bacteraemia is a very serious condition as this results in severe skin lesions and a gangrenous area, a direct sign of the breakdown of epithelial and endothelial tissues by *Pseudomonas,* to access the bloodstream. Fifty per cent of such infections are caused by multi-resistant strains (Tam *et al.*, 2010).

Multi-resistant *Pseudomonas* can also cause pneumonia in patients, whether nosocomial or acquired through exposure to a ventilator. This may also cause chronic lung infections, and severely putting patients with cystic fibrosis (CF) at risk of dying from multi-resistant *Pseudomonas aeruginosa* (Miko *et al*., 2019). Other infections caused by this bacterium include folliculitis (Olszewski *et al.*, 2017), keratitis (Saraswathi & Beuerman, 2015), urinary tract infections (Cole *et al.*, 2014) as well as diabetic foot ulceration (Syafril, 2018).

The pathogenesis in *P. aeruginosa* is attributed to several virulence factors that only become active when needed to cause the several conditions mentioned above. These virulence factors are controlled via QS and play crucial roles in motility, biofilm formations, immune evasion, cytotoxicity and iron scavenging (Lee & Zhang, 2015). Quorum sensing, as well as some of these virulence factors (biofilms, motility, elastase and pyocyanin), will be briefly discussed in the next sections.

2.3. Quorum sensing systems in *Pseudomonas aeruginosa*

2.3.1. Overview

Many bacterial species coexist in a specific environment and must always compete for the available space and nutrients. To dominate and colonize an environment, bacteria interact with one another using specific signals known as acyl-homoserine lactones or AHLs. All AHLs share a common structure that comprises an homoserine lactone ring followed by a carbon chain that vary in length between 4 and 18 carbons (Churchill & Chen, 2011). QS was firstly described in the Gram-negative bacterium of *Vibrio spp* in which LuxI, an AHL synthase, could trigger the activation of bioluminescence genes. The LuxI/LuxR model is used commonly to explain QS in Gram-negative bacteria **(figure 2.1.).** In this figure, an AHL synthase, LuxI, produces the signal molecule (AHL). The AHL diffuses outside the bacterial cells and accumulates in the extracellular environment at high cell density. The signal then diffuses back inside the bacterial cells, where it binds to the response regulator LuxR which then activates target genes that are necessary for the production of virulence factors.

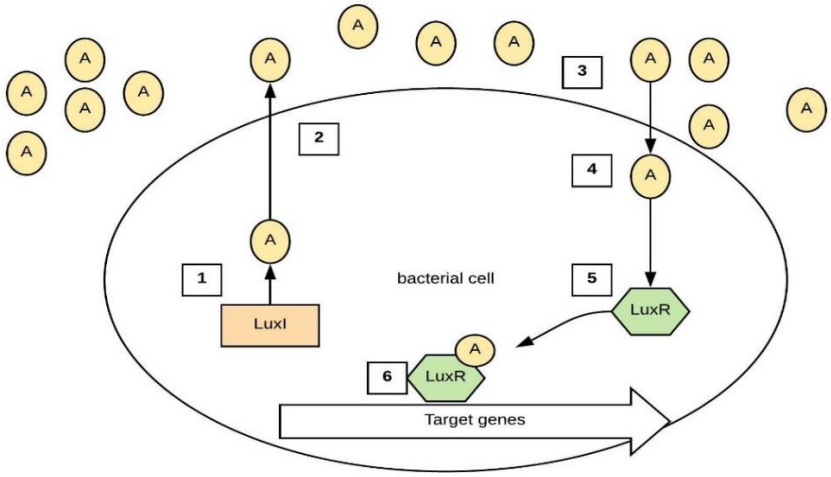

Figure 2.1. Quorum sensing in Gram-negative bacteria. LuxI produces (1) the autoinducer (A) that diffuse outside the cell at low cell density (2). At high cell density, the autoinducer accumulates in the exterior until it reaches a threshold concentration (3). The AIs diffuse back (4) into the cell and bind the response regulator LuxR (5) that activate the QS-regulated target genes (6). Once the target genes are activated, the bacteria can now cause the disease to its host by making use of the virulence factors produced from the activated target genes. Drawn with Diagrams.net (formerly draw.io).

In Gram-negative *Pseudomonas aeruginosa*, at least four QS systems exist and are interconnected: *las*, *rhl*, *pqs* and *iqs* (Lee & Zhang, 2015) **(figure 2.2)**.

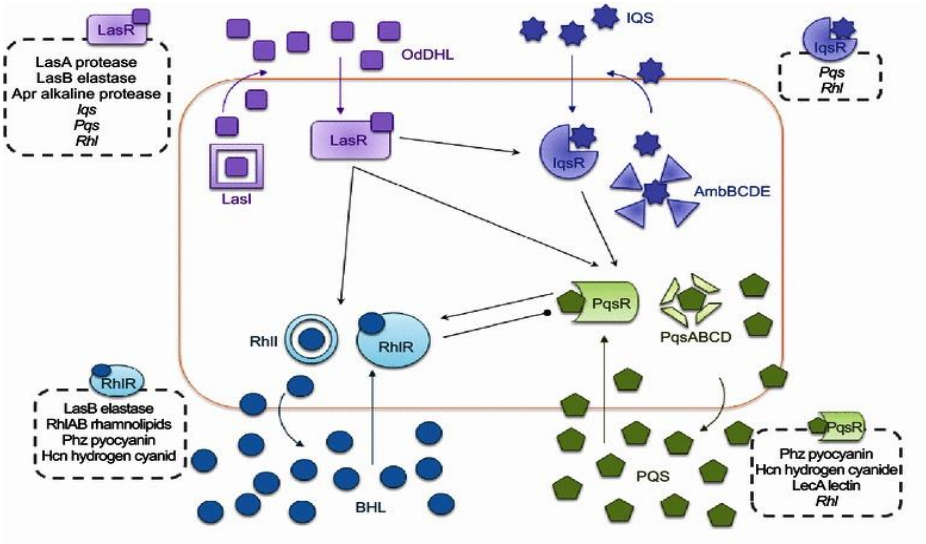

Figure 2.2. The four interconnected quorum-sensing systems (*las, rhl, pqs and iqs*) in *Pseudomonas aeruginosa* (Lee & Zhang, 2015) arrows indicate upregulation; dots indicate downregulation.

In this figure LasI produces the OdDHL signals that diffuses back into cells to bind LasR. The LasR-OdDHL complex activates the *las* system and *rhl* system to activate virulence factors such as proteases, elastases, rhamnolipids, pyocyanin. The *rhl* system is also activated by the synthesis of N-butanoyl-L-homoserine lactone (BHL) that further binds RhlR to activate virulence genes. The *las* system can stimulate the *pqs* system to activate virulence genes such as those for pyocyanin synthesis. In the other hand, the *pqs* system can stimulate the *rhl* system, however rhl can inhibit the expression of PQS. The *las* system also activates the IQS system but only under phosphate stress.

2.3.2. The *las* system

In this interconnected system **(figure 2.2)**, *las* acts as the master regulator for all other systems (*rhl*, *pqs* and *iqs*). The expression of virulence factors is regulated by the synthesis of an AHL molecule by LasI. The response regulator, LasR, gets activated by its AHL molecule, N-3-oxo-dodecanoyl homoserine lactone (3OC12-HSL), and

form a complex *lasR*-3OC12-HSL that activates the *lasI*, *rhlI* and *rhlR* (Lee & Zhang, 2015). The activation of lasI allows for the continuous synthesis of the AHL molecule to form more *lasR*-3OC12-HSL complexes to enhance the activation of the *rhl* system. The genes activated through the *las* system are *lasA* (protease precursor) (Toder *et al.*, 1994, Nadal Jimenez *et al.*, 2012), *lasB* (metalloprotease elastase) (Gambello & Iglewski, 1991), *lasI* (autoinducer), *apr* (alkaline protease) (Gambello *et al.*, 1993) and *toxA* (exotoxin A) (Pesci *et al.*, 1997). The *las* system can also upregulate the *rhl* pathway as well as the *pqs* and *iqs* systems (Lee & Zhang, 2015).

2.3.3. The *rhl* system

The *rhl* synthase (RhlI) synthesizes butyryl-HSL, a C4 AHL molecule that binds to *rhlR* to promote the formation of the *rhlR*-AHL complex. This complex activates the expression of RhlI, causing the enhanced activation of the *rhl* system. The virulence factors activated through this system are factors involved in the production of rhamnolipids (*rhlAB*), that aid in immune evasion as well as contributing towards the formation of biofilms (Lee & Zhang, 2015, Wood *et al.*, 2018, Vijayakumar & Ramanathan, 2020). Moreover, this system is also stimulated by the *pqs* system which affects the expression of *rhl*-mediated virulence genes (Lee & Zhang, 2015).

2.3.4. The *pqs* system

The *rhl* and *las* systems also interact with the *Pseudomonas* Quinolone Signal or *pqs* system. The *pqs* system is mediated by *pqsABCD* that forms the AHL 2-heptyl-3-hydroxy-4-quinolone (PQS), from a precursor 2-heptyl-4-quinolone or HHQ (Kostylev *et al.*, 2019). Both HHQ and PQS are needed to bind the response regulator PqsR, at high cell density to form the PqsR-PQS complex. This complex triggers the expression of several virulence genes such as those also involved in pyocyanin production (Lee & Zhang, 2015). The *pqs* system also interacts with *las* and *rhl* in which the *las* system upregulates *pqs* allowing the expression of pqs-mediated virulence genes. In the other hand, *pqs* is inhibited by *rhl*, this demonstrates that the *pqs* system is dependent on

the concentration ratio of 3OC12-HSL (AHL for *las*) and butyryl-HSL (AHL for *rhl*) in order to be active or not (Lee & Zhang, 2015).

2.3.5. The *iqs* system

The *iqs* system is the recently discovered fourth QS system in *P. aeruginosa* (Lee & Zhang, 2015, Kim et al., 2019). The signal molecule used in *iqs* is 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (IQS) that is produced from *amb*BCDE (Castañeda-Tamez *et al.*, 2018). The signal IQS, at high cell density, binds to the response regulator IqsR to form the IQS-IqsR complex, which activates the *pqs* and *rhl* systems. The *iqs* system is tightly regulated by *las* but only in specific conditions, such as phosphate starvation, *iqs* can take over the *las* system in this case (Kim *et al.*, 2019). In this situation, the *iqs* system displays its ability to upregulate both *rhl* and *pqs* systems under phosphate stress. This, therefore, means that even without a functional *las* system in *Pseudomonas aeruginosa*, *rhl* and *pqs* would still be able to function to activate virulence genes to cause diseases (Lee & Zhang, 2015).

The interconnections between these systems highlight how flexible *P. aeruginosa* can become to overcome adverse effects coming from the environment or the host immune response. The pathogen can use several systems to quickly change and adapt to exploit the niche, in which it is present.

2.4. Pathogenesis and virulence factors in *Pseudomonas aeruginosa*

As aforementioned, *Pseudomonas aeruginosa* expresses its virulence factors via QS and its four interconnected systems. Here, the virulence factors such as biofilm formation and the virulence factors involved in motility (flagella, type IV pili), cytotoxicity (pyocyanin) as well as immune evasion (elastase) are reviewed.

2.4.1. Biofilm formation

Pseudomonas aeruginosa can form complex structures known as biofilms through quorum sensing. The principal composition of biofilms is the extracellular matrix that not only shields the bacterial cells from antibiotics and other environmental factors (e.g., pH, desiccation) but also provides proteins, minerals and important nucleic acids to the cells present (Limoli *et al.*, 2015). Biofilms cause serious medical challenges as they can grow on medical equipment resulting in nosocomial infections and complications in patients that are already affected by CF or HIV.

Biofilms are formed sequentially (Unosson, 2015) as shown in **figure 2.3**.

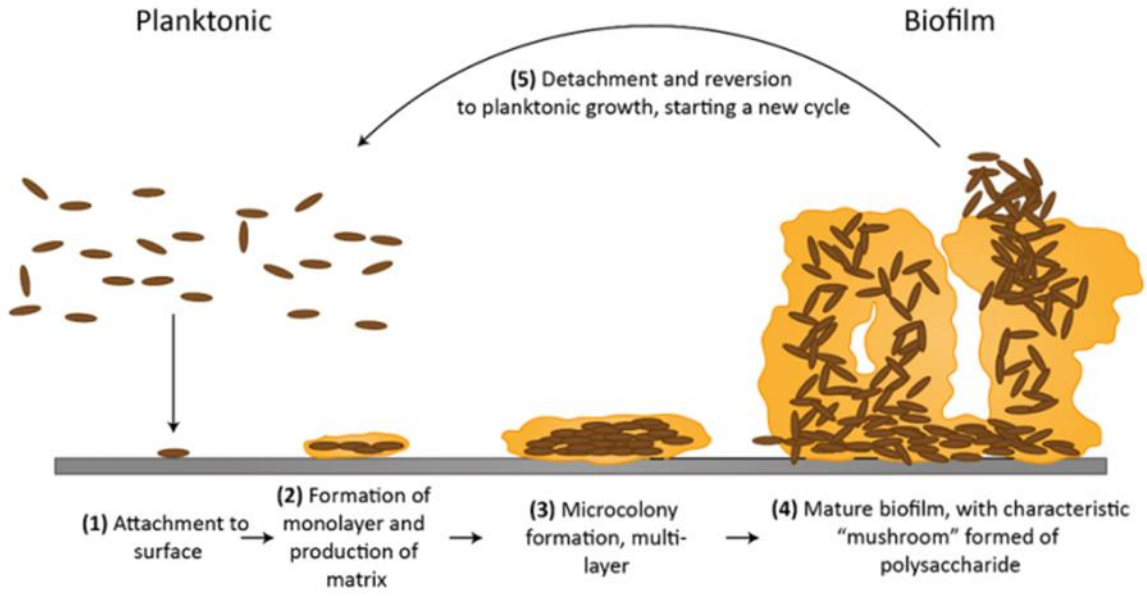

Figure 2.3. **Schematic representation of biofilm formation (Unosson, 2015)**. The initial step involves the reversible attachment of planktonic cells to a surface (1). Once attached, the microcolonies excrete the extracellular matrix (2). Next, the biofilm grows and becomes multi-layered (3), mature and exhibit a characteristic mushroom shape (4). Finally, some cells detach from the sessile structure to form free floating planktonic cells that disperse and able to colonize new surfaces where new biofilm structures will be established.

It begins with the adhesion of free-floating cells to a surface (1). Following this adhesion, microcolonies are formed and start excreting the extracellular matrix (2) which results in the formation of a mature biofilm (3). Furthermore, the mature biofilm grows larger and provides antibacterial properties to the cells in the structure as well as developing a characteristic mushroom structure (4). Finally, new planktonic cells are released from the sessile biofilms and are spread to colonize new surface to form new subsequent biofilms (5). The systems involved

in biofilm formation is *rhl*. This system involves the production of rhamnolipids that are involved in the formation of microcolonies at the first stage of biofilm formation (Rasamiravaka *et al.*, 2015). Rhamnolipids also aid in maintaining the biofilms' structure, thus playing a vital role in the efficiency of the biofilms in *P. aeruginosa* (Pamp & Tolker-Nielsen, 2007).

Therapies to eradicate biofilms are a challenge as the drugs cannot penetrate the extracellular matrix making it crucial to find new ways of dealing with biofilms. New alternatives to fight against biofilms is to target the cell attachment to prevent the initial phase of biofilms formation.

2.4.2. Motility

To occupy a surface or a site inside a host, the pathogen needs to perform swimming motility (translocation in liquid medium), twitching motility (translocation on solid medium) or swarming motility (translocation on semi-solid medium). *Pseudomonas aeruginosa* can perform these types of translocations by using its polar flagellum and type IV pili (O'May & Tufenkji, 2011). Swarming motility is required in the early stages of biofilm formation to initiate the process (Rasamiravaka *et al.*, 2015). Twitching motility is useful in forming microcolonies in *P. aeruginosa* biofilms formation (O'Toole & Kolter, 1998). Both twitching and swarming motility are regulated via the *rhl* system. On the other hand, swimming motility only requires movements flagellar movement (Ha *et al.*, 2014). Moreover, the type IV pili and flagella are useful beyond translocation: they also help in bacterial adhesion and attachment to a surface, prior to the initiation of biofilm formation (Marko *et al.*, 2018).

Disrupting the motility in *P. aeruginosa* could prevent the adhesion of bacterial cells to a new surface and reduce the potential of biofilm formation.

2.4.3. Pyocyanin

Pyocyanin is a blue redox secondary metabolite, synthesized from chorismate in a metabolic pathway involving several enzymes encoded by *phz*ABCDEFGHMS genes that are regulated by the *pqs* system (Lau *et al.*, 2004). Pyocyanin is toxic to the host cells as it causes the production of reactive oxygen species (ROS) such as hydrogen peroxide (Hall *et al.*, 2016, Castañeda-Tamez *et al.*, 2018). It is responsible for the burn wound infections as well as the

acute lung infections. Furthermore, the production of these ROS by pyocyanin is also toxic to other competitors and social cheaters (Castañeda-Tamez *et al.*, 2018). This facilitates the colonization of a suitable surface or site. Pyocyanin is also involved in the release of extracellular DNA (eDNA) which also facilitates the formation of biofilms structure (Das *et al.*, 2016).

Pyocyanin is important in *P. aeruginosa* and preventing the formation of this compound can save countless lives via the reduction of cell lysis in infected patients as well a reduction in biofilms formation.

2.4.4. Elastase

Elastase is a metalloprotease produced by *P. aeruginosa* via the *las* system. The protease plays a vital role in host immune system evasion as highlighted by Kuang *et al.* (2011), van der Plas *et al.* (2016) and Casilag *et al.* (2016). Elastase is also responsible for breaking down the elastin in the host, causing invasion and infections in the host (Spencer *et al.*, 2010). Furthermore, the elastase production allows the pathogen to avoid the host immune system of the host by degrading the complement system as well as IgA and IgG (Li *et al.*, 2019).

Targeting elastase production will prevent the immune evasion of producing *Pseudomonas* cells, it will also prevent the invasion of host cells. Additionally, targeting the *las* system will prevent the activation of virulence genes from other systems as the *las* system acts as a master regulator *pqs* and *rhl*, consequently reducing virulence.

2.5. Antiquorum sensing (AQS) strategies

Although QS allows bacteria to express essential virulence genes, this cell-to-cell communication can be disrupted, preventing or delaying the expression of virulence from a pathogen. Disruption or inhibition of QS is known as antiquorum sensing (AQS) or antipathogenic or quorum quenching (QQ) (Basavaraju *et al.*, 2016). AQS is considered a viable alternative strategy to eradicate multi-drug resistant pathogens. This is because AQS does not create a selection pressure by actively killing the pathogens. The pathogen will be able to grow but will be rendered avirulent.

AQS can happen in several ways (Basavaraju *et al.*, 2016, Asfour, 2018): inhibition of

the AIs synthesis (i), degradation of the AHL signal molecules (ii), inhibition of the AHL molecules transport and inhibition (iii) of AHL binding to the target receptors (iv). The prevention of binding to target receptors (iv) is the most likely strategy to work as this creates a competitive binding event, at the response regulator, between the required AHL molecule and a AQS compound analogue. This involves structural similarities.

To interfere with this binding, analogue molecules of the AHLs/AIs can serve this purpose (Guo *et al.*, 2013). Analogue compounds have a similar structure to the original compound that is usually recognized but differ in the composition, therefore causing a different response in comparison with the standard response. This type of inhibition was demonstrated by Zhu *et al.* (1998) with analogues of 3-oxo-C8-HSL in *Agrobacterium tumefaciens* and Fong *et al.* (2017) with ajoene analogues in *P. aeruginosa*.

Equally important, the ideal AQS compound has been defined, according to Asfour (2018), as being a chemically stable molecule with a low molecular weight that possesses a high specificity with the AHL signal molecule. Moreover, this compound must not cause any toxic side effect on the eukaryotic host as well as on the pathogenic bacterium.

AQS is therefore a different approach that shifts away from the conventional use of antibiotics to treat bacterial infections.

2.6. Sources of antiquorum sensing agents

Plants produce various compounds that vary from alkaloids to terpenoids and flavonoids. These natural compounds create significant interest in research as they have the potential to be used in therapies.

For many thousands of years, humans have faced several diseases and illnesses, to treat them, humans utilized plant materials (such as tree barks, seeds, and leaves or herbs) available in the surrounding environment (Petrovska, 2012). The search for effective plant against a particular disease was based on lay-man's terms or indigenous knowledge as no education was available on the cause of the disease. With the progress made in sciences, it is possible not only to determine the cause of diseases more accurately, however to also look for the exact mechanism of action of a cure.

Plants and herbs are known to produce various bioactive secondary metabolites. It is reasonable to assume that they possess compounds that have AQS properties and that fit the description of the ideal AQS compound. While most studies focused solely on the antimicrobial aspect of plants or herbs, there is a growing interest in researching for novel compounds with AQS properties.

The first AQS compound was discovered in the marine microalga *Delisea pulchra* by Rasmussen *et al.* (2000). The researchers found that halogenated furanones produced by the algae prevented the biofilm formation and the swarming motility of mixed cultures of *Serratia liquefaciens* MG1. These findings were the first evidence that AQS compounds had the potential to be used against bacteria without creating a selection pressure that usually results in bacterial resistance.

2.7. Herbs with AQS properties

Niu *et al.* (2006) focused their work on the compound cinnamaldehyde (CA) produced in cinnamon (*Cinnamomum* genus), and that is responsible for the odour and flavour in cinnamon. They studied the potential anti-quorum sensing of CA using *Vibrio harveyi* strains as biosensors. The results from Niu *et al.* (2006) demonstrated that the

compound CA could interfere with QS by competing with the receptors of two AHLs molecules (3-oxo-C6-homoserine lactone and 3-hydroxy-C4-HSL) as well as inhibiting the autoinducer-2 (AI-2) that is also regulated by QS.

Vattem *et al.* (2007) worked on the potential AQS properties of several herbs and spices including *Origanum vulgare* (oregano)*, Rosmarinus officinalis* (rosemary)*, Ocimum basilicum* (basil)*, Thymus sp* (thyme)*, Brassica oleracea* (cabbage)*, Curcuma longa* (turmeric)*, Zingiber officinale* (ginger) against CV026 and the *C. violaceum* WT (CV31532) as biosensors. The findings indicated the herbs and spices were effective at inhibiting the QS-regulated violacein production, with *Ocimum basilicum* (basil) having the highest inhibition of 78%. Additionally, the herbs and spices reduced the swarming motility of both *E. coli* O157:H7 and *P. aeruginosa* PAO1. The extracts interfered with the QS either by altering the enzymes involved in the conversion of a precursor of an autoinducer molecule or by binding to the autoinducer receptors competitively or non-competitively as suggested by Hentzer & Givskov (2003).

More studies investigated the potential of herbs and spices in AQS. Cosa *et al.* (2019) highlighted the importance of herbal extracts as potential sources of AQS compounds. In this study, sedanolide, a compound in *Apium graveolens* (Celery), reduced the QS-regulated violacein production in *Chromobacterium violaceum*. The compound was identified through GC-MS and provided strong evidence of the great potential of herbs to provide new strategies to fight bacterial infections.

The examples mentioned above indicate that herbs can be good candidates to discover new compounds with AQS activities. This area of research is promising to make a move away from traditional antibiotics. Research focusing on antiquorum sensing will elaborate on the mechanisms of action of these specific AQS compounds.

2.8. Herbs of interest

2.8.1. *Apium graveolens* (Celery)

Apium graveolens or celery belongs to the Apiaceae family and is a perennial plant. It is a popular food flavouring agent but it can be used as a therapeutic agent to treat joint illnesses, gout, arthritis, rheumatisms (Al-Asmari *et al.*, 2017). *A. graveolens* is

also known to display anti-inflammatory properties, anti-cancer properties as well as antimicrobial properties (Khalil *et al.*, 2015). Celery has already proven to be effective in quorum sensing studies. Cosa *et al.* (2019) identified a specific compound in celery that was responsible for inhibiting the violacein production in *C. violaceum*. This compound was sedanolide (**figure 2.4A**), a structural analogue of N-hexanoyl-L-homoserine lactone (C6HSL) (**figure 2.4B**) signal molecule that binds the response regulator (CviR) in this bacterium. The competitive binding of this analogue to CviR prevents the activation of the genes involved in synthesizing violacein, a purple pigment regulated by quorum sensing. This recent study highlights the importance of exploring celery as a potential source of more AQS compounds.

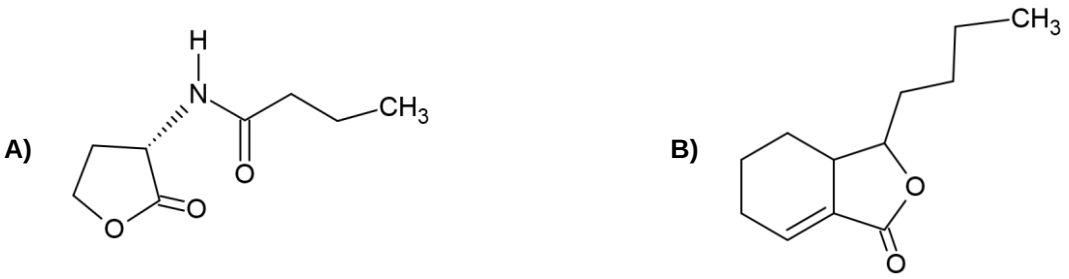

Figure 2.4. Structure of sedanolide **(A)** and N-hexanoyl-L-homoserine lactone (C6HSL) **(B)** (Drawn with ACD/ChemSketch Freeware)

2.8.2. *Coriandrum sativum* (Coriander)

Coriandrum sativum or coriander is an herb that belongs to the Apiaceae family. Coriander is widely used for culinary purposes as well as medicinal purposes. The major compound of this herb is linalool (Sahib *et al.*, 2012). Additionally, there are between seventeen to fifty-two compounds that make the essential oils of this herb, depending on the geographical location. Coriander can be used to lower bad cholesterol, cure mouth ulcers, and reducing menstrual disorders (Satya Shree *et al.*, 2017, Prachayasittikul *et al.*, 2018). Coriander also possesses antibacterial, antioxidants, antidiabetic, anti-inflammatory properties (Sahib *et al.*, 2012). The major compounds in coriander are E-2-decenal (**figure 2.5A**) and linalool (**figure 2.5B**) (Mandal & Mandal, 2015, Chahal *et al.*, 2017). Inhibition of quorum sensing happens

by competitive binding to LuxR type of receptor such as LasR or RhlR, in *P. aeruginosa* (Brackman et al., 2011).

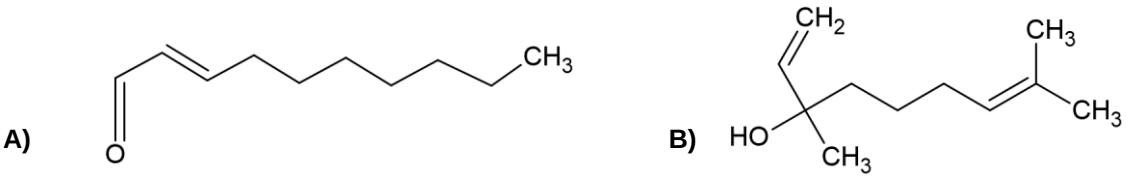

Figure 2.5. Structure of E-2-decenal **(A)** and linalool **(B).** (Drawn with ACD/ChemSketch Freeware)

2.8.3. Rosmarinus officinalis (Rosemary)

Rosmarinus officinalis or rosemary belongs to the Lamiaceae family. This family is popular for its bioactive molecules. Furthermore, rosemary is also used for culinary purposes as well as medicinal purposes to treat wounds, cure asthma, ulcers and also fulfil a role in cholesterol regulation (Andrade *et al.*, 2018). *R. officinalis* has over a hundred different compounds with the most abundant being camphor (**figure 2.6**). Rosemary compounds work in synergy which makes it challenging in determining which compound provides AQS activity (Camele *et al.*, 2019). These compounds also provide antibacterial, antitumor, antioxidant properties (Andrade *et al.*, 2018).

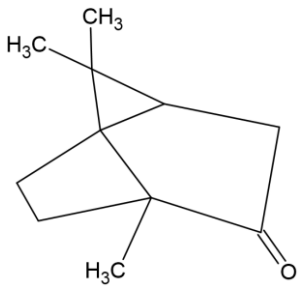

Figure 2.6. Structure of camphor (Drawn with ACD/ChemSketch Freeware)

2.8.4. *Salvia africana lutea* (Dune sage)

Salvia Africana lutea or the brown dune sage is part of the *Salvia* genus which is very popular in terms of medicinal use. The main use of this herb is to treat respiratory ailments such as cold, coughs and bronchitis. It is also used for gynaecological complications. This herb can be administered in herbal tea mixtures or concoctions. The herbs can contain over ninety compounds that provide anti-inflammatory, antimicrobial, antispasmodic properties (Kamatou *et al.*, 2008).

2.8.5. *Salvia officinalis* (Common sage)

Salvia officinalis or the common sage is part of the Lamiaceae family that belongs to the *Salvia* genus. The leaves of this plant contain over forty-nine compounds with 1,8-cineole, α-Thujone and camphor making the majority of the total composition. Additionally, *S. officinalis* possesses antibacterial, antioxidants, antidiabetic and anticancer properties (Sharma & Fagan, 2019) and can also be used to treat chronic bronchitis, rheumatisms, sexual disfunction as well as mental diseases such as Alzheimer (Lemle, 2018).

2.8.6. *Syzygium aromaticum* (Clove)

Syzygium aromaticum or clove belongs to the Myrtaceae family. *S. aromaticum* is used in the food industry as preservatives due to its antioxidant and antimicrobial properties. Additionally, its essential oils are also used for medicinal purposes in dental care, and the treatment of burns and wounds (Batiha *et al.*, 2020). *S. aromaticum* possesses over thirty different compounds, with eugenol being its major compound (Batiha *et al.*, 2020). Rathinam *et al.* (2017) demonstrated the mode of action of eugenol (**figure 2.7A**). This compound was able to interfere with the *las* system in *P. aeruginosa* by competitively binding to the response regulator LasR (inhibits binding of 3OC12-HSL (**figure 2.7B**). The binding highly suggests a certain degree of structural similarities between 3OC12-HSL and eugenol.

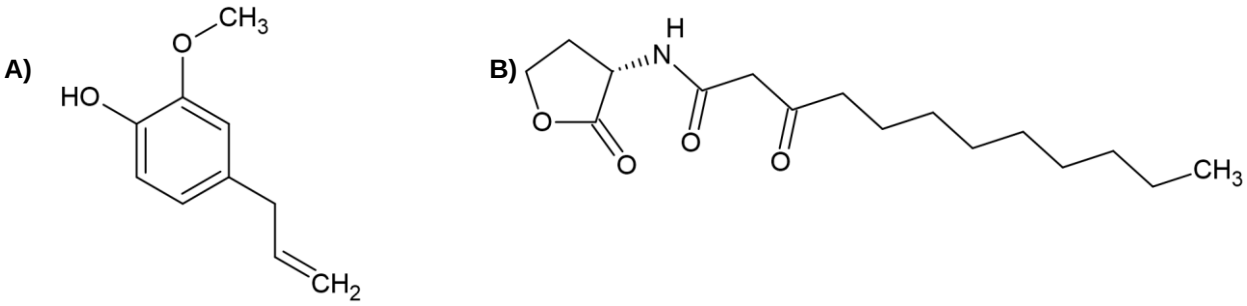

Figure 2.7. Structure of eugenol and C12HSL (Drawn with ACD/ChemSketch Freeware)

2.8.7. Thymus vulgaris (Thyme)

Thymus vulgaris or thyme also belongs to the Lamiaceae family. This herb is used for culinary and medicinal purposes. The main constituents of this herb are thymol and carvacrol. The herb displays various properties such as antimicrobial, anti-inflammatory and antioxidant properties (Almanea *et al.*, 2019) and can also be used to fight dental plaques, gastrointestinal disorders as well as improving liver functions (Reddy, 2014). Thymol (**figure 2.8**) is the major compound in thyme (Cáceres *et al.*, 2020). This compound has proven effective in quorum sensing studies by inhibiting with the violacein production in *C. violaceum* 026 (CV206) by interfering with the consumption of free AHL by CV026 (Myszka *et al.*, 2016). This suggests that thymol can compete with C6HSL to bind to CviR regulator.

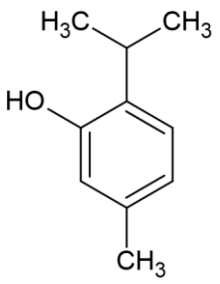

Figure 2.8. Structure of thymol (Drawn with ACD/ChemSketch Freeware)

2.9. Research Gap

The relationship between QS and virulence can be exploited to fight bacterial infections. Novel compounds that can interfere with QS represent a promising area for research. These new compounds can be derived from herbs and can turn out to be more cost-effective compared to synthetic drugs. However, research still needs to be intensified to know precisely the AQS mechanisms of actions of plant compounds and the effective dose that can be used safely to combat bacterial infections worldwide.

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CHAPTER 3 - ANTIBACTERIAL AND ANTIPATHOGENIC PROPERTIES OF SELECTED CULINARY HERBS AGAINST *PSEUDOMONAS AERUGINOSA*

3.1. Introduction

Culinary herbs are used for food flavouring and form an essential part of our diet. Herbs can be used as a substitution to salt and can enhance the flavour of meat and sauces. They are also used in the preservation of food by retarding microbial growth (Cosa *et al.*, 2019). There is a growing interest in literature focusing on the benefits of these culinary herbs that have been used in traditional medicine. They provide several health benefits and are rich in phytochemicals ranging from compounds providing antibacterial properties, anti-cancer properties, anti-inflammatory properties and many more benefits (Guiné & Gonçalves, 2016). Controlling bacterial infections is proving to be a daunting task as the overuse and misuse of antibiotics widely contribute towards the emergence of multidrug-resistant bacterial pathogens such as *Pseudomonas aeruginosa.* Such MDR pathogens cause serious infections through the formation of very strong biofilms and exchanging resistance factors. One famous process that contributes largely to these biofilm formations is quorum sensing (QS).

As afore mentioned, QS allows for the regulation of several processes such as secretion systems, biofilm formation or the activation of virulence genes that allow bacteria to cause diseases inside a host (Pena *et al.*, 2019). Interfering with this QS is a plausible approach towards coming up with new alternatives to antibiotics or antibiotics with unique mechanisms of action. Disparate to antibiotics, AQS compounds do not actively kill the bacteria, as such this prevents the development of antibiotic resistance in pathogenic bacteria. Since then, extensive research in discovering novel compounds that can be found naturally in plants to overcome the burden of MDR pathogens cause each year.

Culinary herbs represent ideal alternatives for therapeutical use as they are well reported and known for providing antioxidant, antimicrobial, antipathogenic properties. They are less likely to be toxic to patients, they are abundant, accessible, and most importantly, culinary herbs are relatively affordable. For these reasons, studies on screening culinary herbs are a good initiative to come up with novel compounds to fight bacterial infections.

This chapter, therefore, focuses on seven selected culinary herbs that were tested to validate their antibacterial potential and screened for their antiquorum sensing activities in order to evaluate their potential as alternatives to antibiotics.

3.2. Material and Methods

This study followed a specific workflow. The first step was to acquire the herbs samples. From this step, extracts were made from the plant samples, with various solvents. The resulting mixtures were later screened to determine the active extracts in order to narrow the scope of the study.

3.2.1. Plant materials

The herbs samples, *Apium graveolens* (celery), *Syzygium aromaticum* (clove), *Coriandrum sativum* (coriander), *Rosmarinus officinalis* (rosemary), *Salvia Africana lutea* (brown dune sage), *Salvia officinalis* (common sage) and *Thymus vulgaris* (thyme) were purchased from Eat Your Greens farm located in Midrand, South Africa, in November 2018. . The criterion for choosing these herbs was based on their traditional use in medicine (**table 3.1**).

Table 3.1. Culinary herbs investigated for their potential AQS properties

Species name	Family name	Common name	Medicinal uses	Reference
Apium graveolens	Apiaceae	Celery	Treat joint illnesses or gout, flu, colds, high blood pressure. Possesses anti-inflammatory, anti-cancer, antimicrobial properties	Singh & Handa (1995), Covington (2001), Al-Asmari *et al.* (2017)
Coriandrum sativum	Apiaceae	Coriander	Lowers bad cholesterol, cure mouth ulcers, reduces menstrual disorders, antibacterial, antioxidants, antidiabetic, anti-inflammatory properties	Satya Shree *et al.* (2017), Prachayasittikul *et al.* (2018)
Rosmarinus officinalis	Lamiaceae	Rosemary	Antibacterial, antitumor, antioxidant properties, asthma attenuation, cholesterol regulation, antiulcer properties	(Andrade *et al.*, 2018)
Salvia africana lutea	Lamiaceae	Dune sage	Cold, coughs, bronchitis, anti-inflammatory, antimicrobial, antispasmodic properties	(Kamatou *et al.*, 2008)
Salvia officinalis	Lamiaceae	Common sage	Mental diseases (e.g., Alzheimer), fever, rheumatisms, sexual malfunction, chronic bronchitis, antibacterial, antioxidants, antidiabetic and anticancer properties	(Almanea *et al.*, 2019), (Lemle, 2018)
Syzygium aromaticum	Myrtaceae	Clove	Dental care, headaches, sore throat, antidiabetic, antioxidant and antimicrobial properties	(Ajiboye *et al.*, 2016), (Batiha *et al.*, 2020)
Thymus vulgaris	Lamiaceae	Thyme	Fight dental plaques, cure gastrointestinal disorders, improve liver function, laryngitis, antimicrobial, anti-inflammatory and antioxidant properties	(Karabegovic *et al.*, 2012, Satyal *et al.*, 2016) (Reddy, 2014)

3.2.2. Extraction method

The extraction method used was similar to (Cosa *et al.*, 2019). The herbs were dried on the lab workbenches for 2-4 days at room temperature. The dried herbs were later ground to fine powder using a grinder (IKA2870900 MF 10.1, Cole Palmer Scientific Experts, Chicago, USA). The powders were later used for extraction using hexane, ethyl acetate, methanol and dichloromethane (DCM) as solvents. The ground powders (30.0 g) were mixed with the solvents (350 ml) and incubated with a shaking apparatus (Labcon, Krugersdorp, South Africa) at 160 rotations per minute (rpm) for 48 hours. The resulting crude extracts were later filtered using Whatman No. 1 paper filters (Munktell filters, Ahlstrom Munksjö, Helsinki, Finland) into 10 ml polytop vials (Lasec, Johannesburg, South Africa) and dried in a fume hood (Vivid Air, Pretoria, South Africa) for 7-14 days until the solvent had completely evaporated.

For aqueous extracts, extraction was performed by mixing 30.0 g of ground powder with 350 ml of sterile distilled water. The contents were boiled for 50 minutes using a hotplate (Chemlab Supplies, Midrand, South Africa) and filtered into vials (Lasec, Johannesburg, South Africa) using paper filters (Munktell filters, Ahlstrom Munksjö, Finland). The crude extracts were cooled down at room temperature and allowed to dry for 7-14 days inside an oven (EcoTherm, Labotec, Johannesburg, South Africa).

All extracts were later dissolved in 1% dimethylsulfoxide (DMSO) (Merck, Johannesburg, South Africa), stored inside polytope vials (Lasec, Johannesburg, South Africa) to yield stocks of 32.0 mg/ml, for further assays. All extracts were preserved at 4°C in a Labocool fridge (Labotec, Johannesburg, South Africa) for downstream applications.

3.2.3. Determination of the minimum inhibitory concentration (MIC) of the extracts on *P. aeruginosa*

The MIC of the extracts was assessed using the broth microdilution assay (Eloff, 1998), with 96-wells microtiter flat-bottom plates on *P. aeruginosa*. Two-fold dilutions were performed from the crude extracts to yield eight different concentrations ranging from 0.0625 mg/ml to 8.00 mg/ml for each **(figure 3.1)**. Firstly, Mueller-Hinton (MH) broth (LabM, United Kingdom) was added (100 µl) to the wells followed by the addition of 100 µl of the extracts to each well of the first row. The serial dilutions were performed by transferring 100 µl of the contents from the first row (row A) to the adjacent wells in the next row (row B) and so on until the last row (row H). Lastly, the bacterial culture, standardized at an OD_{600nm} of 0.1, was added to each well of the experimental samples. In this study, 1% DMSO was used as the negative control, while vanillin (0.50 mg/ml), cinnamaldehyde (0.50mg/ml) and ciprofloxacin (0.10 mg/ml) (Merck, South Africa) were used as positive controls.

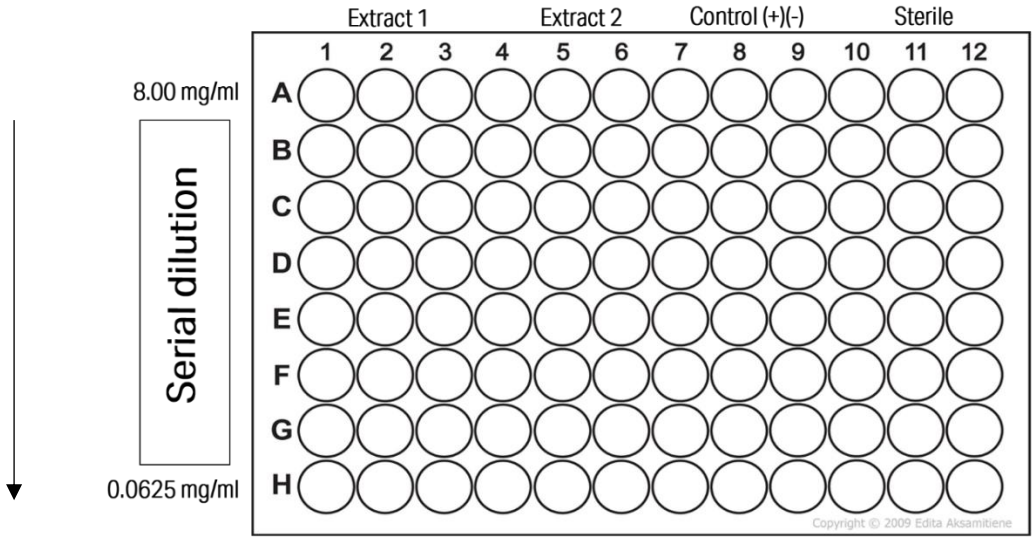

Figure 3.1. Microtiter plate depicting the assay setup. 96-wells plate template obtained from http://www.cellsignet.com/media/plates/96.jpg. Labels drawn with Diagrams.net (formerly draw.io).

The microtiter plates containing the experimental samples were incubated at 37ºC for 24 hours with a shaking incubator at 120 rpm (Labcon, Johannesburg, South Africa). The results were visualized after the addition of 40 µl of 0.20 mg/ml of p-iodonitrotetrazolium violet (INT) (Merck, Johannesburg, South Africa) into each well

and incubating at 37ºC for 1 hour. The MIC was determined as the lowest concentration that prevented the formation of a red colour after the addition of INT.

3.2.4. Screening for AQS activity

3.2.4.1. Qualitative screening

The first part of the screening was performed using the disk diffusion method. *Chromobacterium Violaceum* 12472 (CV12472), obtained from the Centre for Microbial Ecology and Genomics (CMEG) at the University of Pretoria (South Africa). CV12472 produces a purple pigment (violacein) under the control of QS. This bacterium helps in visualizing whether QS (production of violacein) or AQS (inhibition of violacein production) occurs. CV12472 was grown in Luria Bertani (LB) broth overnight and standardized to an absorbance of 0.1 at 600nm, using a spectrophotometer (SpectraMax DU720, Beckman Coulter, South Africa). Sterile disks were inoculated with four different concentrations (0.35, 1.80, 3.90, 7.00 mg/ml) of each crude extract, the positive controls contained cinnamaldehyde (0.50 mg/ml) (Merck, South Africa) and vanillin (0.50 mg/ml) (Merck, South Africa). The dried disks were later placed on LB plates swabbed with CV12472 on their surface. The samples were incubated at 28ºC for 24 hours. The results were obtained by measuring the diameters of zones containing growing cells but with no production of the purple violacein (violacein pigment inhibition). The diameter of the zones of the violacein pigment inhibition was measured with a ruler to classify the activity of the extracts into three categories as described by the Clinical and Laboratory Standards Institute (2020):

- above 20 mm = Susceptible (S)
- 15-19 mm = Intermediate (I)
- below 14 mm = Resistant (R)

3.2.4.2. Quantitative AQS screening

The quantitative screening was performed using 96-well microtiter plates. A bacterial culture of CV12472 was prepared and incubated overnight in LB agar plates. A single colony was grown overnight and transferred into sterile distilled water and standardized to 0.5 McFarland (OD_{600nm}: 0.1). All samples were prepared in triplicates by transferring LB broth (100µ) in all wells and 100 µl of each crude extract in the first row of each plate. Vanillin (0.50 mg/ml), Cinnamaldehyde (0.50 mg/ml), and Ciprofloxacin (Merck, South Africa) (0.10 mg/ml) were used as positive controls whereas 1% DMSO was used as a negative control. Sterile LB broth and standardized bacterium were set as blanks on the microtiter plates.

All samples were diluted resulting in decreasing concentrations ranging from 0.0625 mg/ml to 8.00 mg/ml. Following this, 100 µl of the standardized (OD_{600nm} of 0.4) bacterium was added to every well. The OD_{600nm} was recorded before incubation at 28ºC for 24 hours with shaking (120 rpm). Furthermore, the OD_{600nm} was recorded a second time following the incubation to determine the viability of the bacterial cells. The samples were then dried in an oven (EcoTherm, Labotec, Johannesburg, South Africa) set at 50ºC for 24 hours. Afterwards, the samples were resuspended with the addition of 200 µl of 100% DMSO (Merck, Johannesburg, South Africa). And were again incubated at 28ºC with shaking (120 rpm) for 2 hours to solubilize the violacein. Following that, the absorbance was re-recorded at 485 nm to detect the presence or absence or reduction of violacein. The percentage of violacein inhibition was calculated using the following equation:

$$Percentage\ violacein\ inhibition = \frac{OD585\ control\ - OD585\ experimental}{OD585\ control} \times 100$$

3.2.5. Statistical analysis

Results are presented as a standard deviation that was calculated from the mean values calculated in GraphPad Prism version 8.4.3. One way ANOVA was also used to determine the significance of the assay results. Statistical difference is considered significant if $p<0.05$.

3.3. Results and Discussion

3.3.1. Antibacterial activities of selected culinary herbs

When antibacterial properties of the selected culinary herbs were assessed for minimum inhibitory concentration (MIC), out of thirty-five extracts, only eleven extracts displayed noteworthy antibacterial activity (1.00 mg/ml or less, **table 3.2**) (van Vuuren, 2008). These extracts are *Salvia officinalis* (hexane and DCM), *Rosmarinus officinalis* (hexane and aqueous), *Syzygium aromaticum* (ethyl acetate, methanol, and DCM), *Coriandrum sativum* (ethyl acetate, methanol, hexane and DCM).

Nine other extracts displayed activity at 2.00 mg/ml, we classified as intermediate activity because they have no noteworthy activity (1.00 mg/ml or below) but are still not far from that cut-off value. The extracts with intermediate activity were from *Apium graveolens* (ethyl acetate, DCM and aqueous), *Salvia africana lutea* (aqueous), *Thymus vulgaris* (aqueous), *Salvia officinalis* (ethyl acetate), *Rosmarinus officinalis* (methanol), *Syzygium aromaticum* (hexane) and *Coriandrum sativum* (aqueous). The rest of the extracts did not display any intermediate or noteworthy antibacterial activity whatsoever. The 1% DMSO did not affect the *P. aeruginosa* and the bacterium was able to survive in the set conditions. On the other hand, cinnamaldehyde (0.50 mg/ml), vanillin (0.50 mg/ml) as well as ciprofloxacin (0.10 mg/ml) were able to inhibit the growth of the bacterium strongly as shown in **table 3.2**. Cinnamaldehyde is well known for inhibiting the growth of *P. aeruginosa*, hence the MIC value below 1.00 mg/ml (Cox & Markham, 2007, Tetard *et al.*, 2019). Furthermore, Bezerra *et al.* (2017) demonstrated that vanillin displays MIC values

above 1.00 mg/ml, which is still in line with the results obtained in this experiment as vanillin was able to inhibit *P. aeruginosa* growth at 0.50 mg/ml.

Table 3.2. Minimum inhibitory concentrations values (mg/ml) of selected culinary herbs against *Pseudomonas aeruginosa*.

Herbs	Solvents				
	HEX	**DCM**	**EA**	**MET**	**H_2O**
Apium graveolens	4.00	**2.00**	**2.00**	4.00	**2.00**
Coriandrum sativum	**1.00**	**1.00**	**1.00**	**1.00**	**2.00**
Rosmarinus officinalis	**1.00**	4.00	8.00	**2.00**	**1.00**
Salvia africana lutea	8.00	8.00	8.00	8.00	**2.00**
Salvia officinalis	**1.00**	**1.00**	**2.00**	8.00	4.00
Syzygium aromaticum	**2.00**	**1.00**	**1.00**	**1.00**	4.00
Thymus vulgaris	8.00	8.00	8.00	8.00	**2.00**
1% DMSO	8.00				
Cinnamaldehyde	**0.50**				
Vanillin	**0.50**				
Ciprofloxacin	**0.10**				

HEX = Hexane - DCM = Dichloromethane - EA = Ethyl acetate - MET = Methanol - H_2O = Aqueous

Values in green indicate noteworthy activity (1.00 mg/ml or less) and values in orange indicate intermediate activity (2.00 mg/ml).

All extracts displayed antibacterial activity of 8.00 mg/ml or below, backing the reputation of culinary herbs used in medicine. *Apium graveolens* (celery) is a well-known reported herb with good medicinal benefits. It has extensive use in traditional medicine in curing ailments such as diabetes (Covington, 2001), hypertension (Al-Asmari *et al.*, 2017), asthma, bronchitis and hepatitis (Singh & Handa, 1995). In this experiment, celery does not display noteworthy antibacterial activity at all, but only an intermediate antibacterial activity at 2.00 mg/ml (ethyl acetate, DCM and aqueous) while the methanol and hexane extracts display 4.00 mg/ml MIC values. This result is also aligned with the research from Cosa *et al.* (2019) in which they displayed that

celery extracts have MIC values of 4.00 mg/ml for all the solvents (water, methanol, DCM and ethyl acetate). These findings demonstrate that celery has antibacterial activity and is a culinary herb that can be used in medicine despite not displaying noteworthy activity. Celery is very rich in phenols, alkaloids, flavonoids as well as steroids that confer these properties.

C. sativum (coriander) herbs pair well with many dishes and are commonly used in culinary. Coriander leaves provide various medicinal benefits such as providing anti-inflammatory, antioxidants, antidiabetic properties and many more (Satya Shree *et al.*, 2017, Prachayasittikul *et al.*, 2018). Coriander also displays antibacterial activity as demonstrated by Zangeneh *et al.* (2018) in which they had concentrations of coriander ethanolic extracts at 2.00 mg/ml that displayed antibacterial activity against *P. aeruginosa* (ATCC 27853).

R. officinalis (rosemary) has commonly been used as a condiment and food preservative. Additionally, it can also be used in traditional medicine to attenuate asthma (de Oliveira *et al.*, 2019), to regulate cholesterol (Fernández *et al.*, 2014). This herbs also possesses antiulcer properties (Amaral *et al.*, 2013), anti-inflammatory properties, antioxidant properties (Scheckel *et al.*, 2008) and many more (de Oliveira *et al.*, 2019). In this study, rosemary displayed noteworthy activity with its aqueous and hexane extracts. The methanol extract displayed intermediate activity whereas the EA and DCM extracts displayed antibacterial activities of 8.00 and 4.00 mg/ml, respectively. Comparing to other studies, Cosa *et al.* (2019) also demonstrated the antibacterial activity of this herb with the aqueous and methanol extracts displaying 2.00 mg/ml while the EA and DCM extracts displayed MICs of 4.00 mg/ml. On the other hand, Al Zuhairi *et al.* (2020) demonstrated the aqueous extracts of rosemary have noteworthy antibacterial activity on other Gram-negative bacteria such as *Klebsiella pneumoniae* and *Proteus vulgaris*. These MICs fall within the antibacterial activity range and confirms the use of rosemary in traditional medicine.

S. africana lutea (dune sage) can be used as a substitute for the common sage (*Salvia officinalis*) in cooking. It can be used in dishes containing meat and can also serve as dressing in salads. This herb is also used as a decoction to treat colds, cough and flu (Kamatou *et al.*, 2008). In terms of antibacterial activity, this herb displays MICs of 8.00 mg/ml, except the aqueous extracts (2.00 mg/ml). This translates into *S.*

africana lutea having antibacterial activity against *P. aeruginosa,* in this experiment, with its aqueous extract having intermediate activity. Another study by van Vuuren *et al.* (2019) demonstrated the noteworthy activity of this herb. However, this noteworthy activity was against a gram-positive bacterium species *Brevibacterium linens.*

S. officinalis (common sage) pairs well with any meat to enhance flavour. The common sage is also very popular for providing multiple medicinal benefits that help in treating gout, ulcers, hyperglycemia, diarrhoea as well as displaying anti-inflammatory properties (Ghorbani & Esmaeilizadeh, 2017). In this experiment, sage was good at displaying antimicrobial activity for all the extracts with intermediate activity for the EA extracts. The hexane and DCM extracts displayed noteworthy antimicrobial activity. Adrar *et al.* (2016) conducted their research using the common sage aqueous extracts against several pathogens including *P. aeruginosa* 867 and found that their aqueous extracts displayed noteworthy activity with MICs at 1.00 mg/ml. This is contrasting with the aqueous our findings that did not display noteworthy activity. This difference can be due to the difference in geographical location as the research from Adrar *et al.* (2016) was conducted in Algeria. The difference in geographical location also means a different climate in which the herb was grown. Phyto composition is linked to climate which can affect the composition of compounds within a specific herb species (Liu *et al.*, 2018).

S. aromaticum (clove) leaves are used in culinary as flavouring agents, provide health benefits in humans and can be used for pain relief during dental work, alleviate sore throat as curing respiratory disorders (Ajiboye *et al.*, 2016). Clove also provides antibacterial, antioxidant and antidiabetic properties (Ajiboye *et al.*, 2016). The crude extracts of clove extracts displayed antimicrobial properties; the EA, methanol and DCM extracts showed noteworthy activity whereas the intermediate activity occurred in the hexane extract. In comparison to other studies, Cosa *et al.* (2019) demonstrated that clove displayed an intermediate activity from the EA, DCM, methanol and aqueous extracts. Mostaqim *et al.* (2019) also had a noteworthy antibacterial activity (0.70 mg/ml), against *P. aeruginosa*, for the clove aqueous extracts reinforcing the ability of clove to inhibit bacterial growth.

T. vulgaris (thyme) is extremely popular in culinary for seasoning and enhancing flavour and is also used in alcoholic beverages in liqueurs (Karabegovic *et al.*, 2012).

Thyme is also well known for providing medicinal benefits helping in fighting bronchitis, gastrointestinal distress, helping in getting rid of dental plaques (Satyal *et al.*, 2016) as well providing antioxidant, anti-inflammatory and antibacterial properties (Basch *et al.*, 2004). In this experiment, thyme displayed antibacterial properties against *P. aeruginosa*, however only the aqueous extract had an intermediate activity. The other extracts did not display any noteworthy activity at all. Similar results were obtained from Cosa *et al.* (2019) in which no noteworthy activity was detected for all extracts of thyme (aqueous, DCM, EA and methanol).

The variations observed within this experiment demonstrate that the extracts have a wide range of compounds or mixture of compounds that are responsible for the activities observed. Not all solvents extract the same kind of molecules, hence the use of five different extracts to maximize the range of possible compounds that can be obtained. (Zhang *et al.*, 2018). For extraction of polar compounds, methanol & water are very well-known solvents. For the extraction of non-polar compounds, hexane is a popular solvent. Finally, for mid-polar compounds, ethyl acetate is a well-known solvent and DCM can also be used for that purpose (Abarca-Vargas *et al.*, 2016).

The results obtained from this study often vary with previous work that has been done before. These variations are due to the type of strains used, the composition of the compounds inside the different extracts, the method of extraction, as well as the storage of these extracts. Many of these factors can influence the results which make it complicated to replicate.

Despite the observations made, no conclusions could be made regarding the anti-quorum sensing properties of the extracts. However, the experimental extracts display antibacterial activity across all solvents. These results still allow us to speculate that compounds with AQS activity can also be found. The AQS compounds will not actively kill the bacteria but inhibit their ability to communicate through quorum sensing.

3.3.2. Qualitative measurements of AQS activity

In the preliminary AQS disk diffusion assay, we were evaluating the potential of the selected extracts (concentrations ranging from 0.0625 mg/ml to 8.00 mg/ml) to display AQS properties against the biomonitor *Chromobacterium violaceum* 12472 (CV12472). This bacterium produces a purple pigment, violacein. The effectiveness of the extracts was assessed by the observation and measurements of violacein inhibition zones around the extracts (**table 3.3**).

Syzygium aromaticum (clove) and *Coriandrum sativum* (Coriander) hexane extracts inhibited violacein production at more than one concentration. The clove hexane extracts displayed violacein inhibition activity at 0.35 mg/ml (9.00 mm) as well as 1.80 mg/ml (9.00 mm). Despite this observation, it was assumed that CV12472 was resistant to this specific extract.

The coriander hexane extract displayed violacein inhibition activity at all four concentrations: 0.35 mg/ml, 1.80 mg/ml, 3.90 mg/ml, 7.00 mg/ml with violacein inhibition zones of 16.00 mm, 28.33 mm, 24.66 mm and 28.66 mm, respectively.

T. vulgaris (thyme) ethyl acetate extract (EA) also displayed AQS properties at 7.00 mg/ml (11.00 mm). However, this result translates to CV12472 being resistant to this specific extract. The rest of the thirty-two extracts did not show any AQS activity whatsoever on CV12472 violacein production.

Among the control plates, cinnamaldehyde (0.50 mg/ml) displayed a broad zone of violacein inhibition (26.00 mm) whereas vanillin (0.50 mg/ml) did not show any AQS activity for this assay despite vanillin being a popular and well-reported AQS compound (Choo *et al.*, 2006, Ponnusamy *et al.*, 2009).

Table 3.3. AQS Disk diffusion assays from different extracts against CV12472

Violacein inhibition zones (mm)																				
Extract concentrations																				
Herbs	**0.35 mg/ml**					**1.80 mg/ml**					**3.90 mg/ml**					**7.00 mg/ml**				
	HEX	**DCM**	**EA**	**MET**	**H2O**	**HEX**	**DCM**	**EA**	**MET**	**H2O**	**HEX**	**DCM**	**EA**	**MET**	**H2O**	**HEX**	**DCM**	**EA**	**MET**	**H2O**
Apium graveolens	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Coriandrum sativum	**16(I)**	0	0	0	0	**28.33(S)**	0	0	0	0	**24.66(S)**	0	0	0	0	**26.66(S)**	0	0	0	0
Rosmarinus officinalis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Salvia africana lutea	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Salvia officinalis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Syzygium aromaticum	**9(R)**	0	0	0	0	**9(R)**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Thymus vulgaris	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**11(R)**	0	0
Cinnamaldehyde (0,50 mg/ml)	**26 (S)**																			
Vanillin (0,50 mg/ml)	**0 (R)**																			
1%DMSO	**0 (R)**																			

The zones of violacein inhibitions (in mm) were determined by calculating the average of triplicate measurements of the violacein inhibition diameters

Although *C. sativum* is also known to being popular in antimicrobial species, Alves *et al.* (2016) showed that *C. sativum* displayed some AQS activity by inhibiting the violacein produced by CV12472 with a broad zone of violacein inhibition at 53.00 mm. No recent literature is available concerning *C. sativum* and *S. aromaticum* hexane extracts use for preliminary AQS screening against CV12472. However, the experiment results suggest that violacein was inhibited. The conclusion that *S. aromaticum* and *C. sativum* hexane extracts possess AQS compounds, can be drawn due to the observation that can inhibit violacein at more than one concentration, with the coriander inhibition being concentration dependent whereas clove inhibits violacein smaller concentrations.

Violacein production in CV12472 is mediated by the production of the C6 HSL that activates the vio genes (Kothari *et al.*, 2017). From the results observed, we can determine that the *Syzygium aromaticum* and *Coriandrum sativum* hexane potentially contain a compound or a mixture of compounds that prevents the binding of the C6 HSL to the *vio* genes and therefore interfering with the violacein production in CV12472. These two hexane extracts display AQS properties at lower concentrations and more than one concentration. However, from this preliminary screening, it is impossible to quantify the AQS activity of the two hexane extracts. To accomplish this, a more sensitive assay is needed (Baloyi *et al.*, 2019). This is why the next screening had to focus on the quantitative measurements of the AQS inhibition of both *S. aromaticum* and *C. sativum* hexane extracts.

3.3.3. Quantitative measurements of AQS activity

The quantitative inhibition was performed to determine the amount of violacein that was inhibited by the *S. aromaticum* and *C. sativum* hexane extracts (0.0625 mg/ml to 8.00 mg/ml). After 24 hours of incubation, the clove and coriander hexane extracts did allow for the growth of CV12472 (**figure 3.2A**). Both absorbance readings at 600nm after 24 hours were above 0.4. The cells of CV12472 were still viable after 24 hours of exposure to the hexane extracts. Comparative to the controls such as cinnamaldehyde (0.50 mg/ml) and ciprofloxacin (0.10 mg/ml) displayed the highest

bacterial growth inhibition. On the other hand, vanillin (0.50 mg/ml) did not inhibit the growth of CV12472 after 24 hours (OD_{600nm} above 0.5).

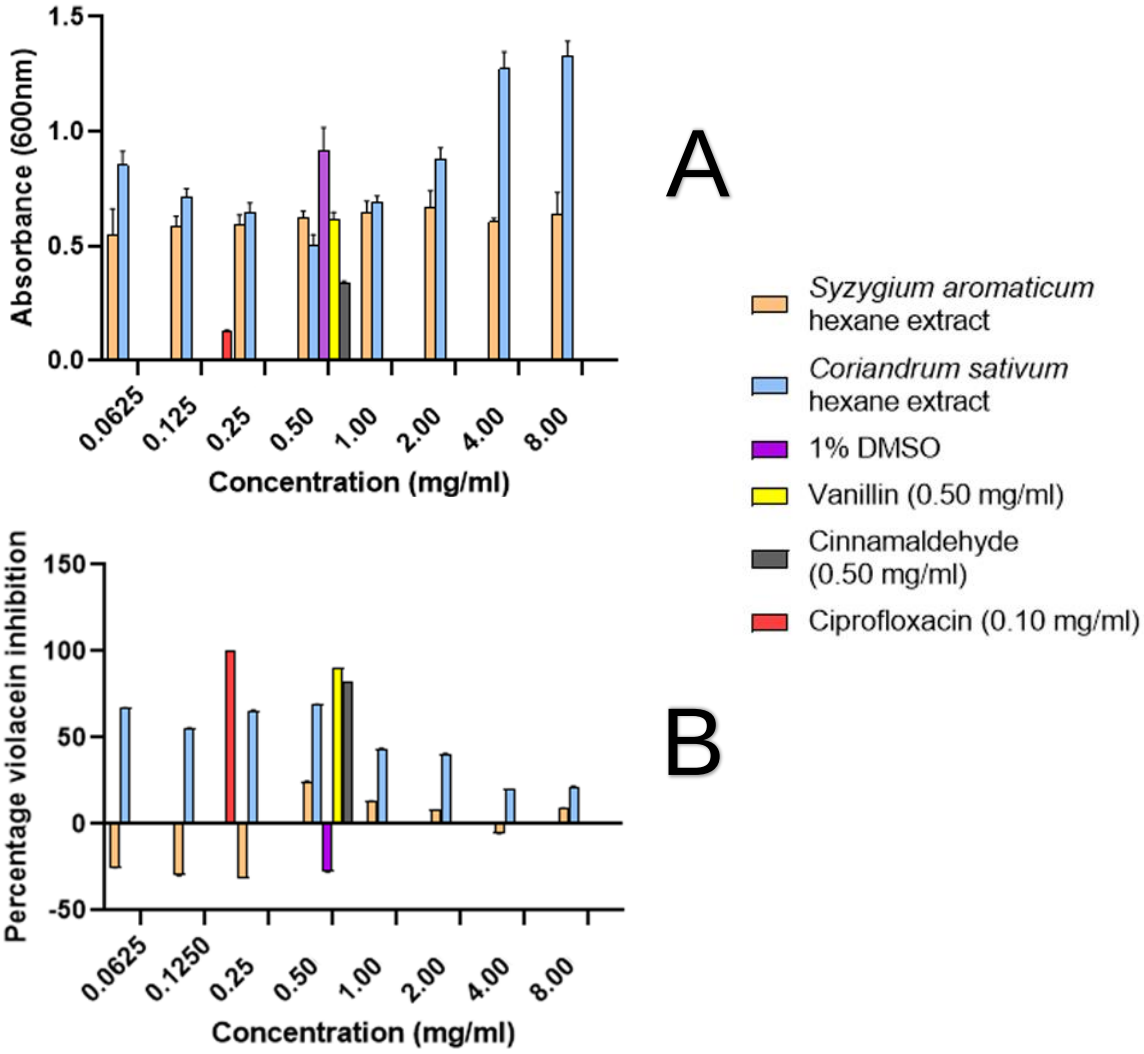

Figure 3.2A. Growth of CV12472 in clove and coriander hexane extracts following a 24 hours incubation. The graph displays the bacterial growth at OD_{600nm} under different conditions. There is significance between the differences observed ($p<0.05$). Ciprofloxacin and cinnamaldehyde strongly inhibit bacterial growth after 24 hours, whereas 1% DMSO and vanillin also allow for bacterial growth after 24 hours. *S. aromaticum* (Clove) and *C. sativum* (coriander) display significant bacterial growth, above OD_{600nm} of 0.5, at all concentrations after 24 hours.

Figure 3.2B. Per cent violacein inhibition of clove and coriander extracts against CV12472. The graph displays percent violacein inhibition at OD_{485nm} under different conditions. There is significance between the differences observed ($p<0.05$). Ciprofloxacin killed all bacterial cells (100% inhibition of violacein due to no viable cells). Vanillin and cinnamaldehyde have strong violacein inhibition of 90% and 82% respectively. The 1% DMSO did not show any negative on violacein production. Both *S.*

aromaticum (clove) and *C. sativum* (coriander) display the highest violacein inhibition at 0.50 mg/ml with 24% and 69%, respectively.

The optical density measurements were taken a second time (48 hours post-incubation) at 485 nm to measure the amount of violacein produced through absorbance readings. The results were later converted to violacein inhibition percentage (Equation 1) to quantify the AQS properties of the clove and coriander hexane extracts (**figure 3.2B**).

The cinnamaldehyde and vanillin control inhibited violacein by 82 and 90% respectively. Ciprofloxacin, strongly inhibited the violacein production. This was likely due to antibacterial activity and therefore, there was no viable cells to produce the pigment. From the experimental results obtained, the *C. sativum* (coriander) extract was active at all concentrations with its highest violacein inhibition at 69% (0.50 mg/ml). Furthermore, the *S. aromaticum* (clove) hexane extract was only active above 0.50 mg/ml but with lower inhibition at a maximum of 24%. Below 0.50 mg/ml, no inhibition was observed for this specific extract (**figure 3.2B**). These results also display the higher AQS efficiency of *C. sativum*, in contrast to the lower AQS activity of the hexane extract of *S. aromaticum*, that was observed in the preliminary screening using CV12472.

These results show that the hexane extract of *C. sativum* is good at inhibiting the violacein production in *C. violaceum* 12472, thus it has AQS properties. The hexane extract of *S. aromaticum* was able to inhibit violacein at 0.50 mg/ml but at a lower reduction (24%). A compound or a combination of compounds in the crude extracts are responsible for this activity. *S. aromaticum* is known to inhibit quorum sensing in other bacterial species such as in *E. coli* [pSB401] (Krishnan *et al.*, 2012), in *Serratia spp*. (Yamarthi *et al.*, 2014) and in yeast cells, like *Candida* (Rajkowska *et al.*, 2019). The low inhibition of violacein from the clove hexane sample could be explained by the extraction method used as well as the composition of the compounds, that is affected by the season and environmental factors like climate (Frohlich *et al.*, 2019). according to literature, in *S. aromaticum*, the major compound is eugenol. This compound explains the AQS activity observed as eugenol is a well-reported AQS

agent (Rathinam *et al.*, 2017) that is found in several other plants (Khalil *et al.*, 2017) and can bind competitively to signalling AHL molecules receptors.

On the other hand, Al-Haidari *et al.* (2016) displayed the AQS activity of coriander as potent and that there is a clear indication of AQS compounds present in the herb. For *C. sativum*, literature premises that two major compounds, namely Linalool and E-2-decenal (Mandal & Mandal, 2015), are responsible for the AQS activity. In *C. sativum* leaves, E-2-Decenal dominate over linalool, in composition. E-2-Decenal is capable of competitively bind to LuxR types of receptors (Brackman *et al.*, 2011). In CV12472, violacein is produced through a LuxI/LuxR homolog system, namely CviI/CviR (Kothari *et al.*, 2017). This entails that the E-2-decenal compound is able to interfere with CV12472 AHL signalling molecule (C6HSL) for the binding to CviR to deactivate the synthesis of violacein. These two hexanes extract of *S. aromaticum* and *C. sativum* show potential AQS properties based on the preliminary screening. These extracts can be used for further analysis to determine the effects they have on *P. aeruginosa* virulence factors.

3.4. Conclusions

From this study, we have used several extracts to screen for their potential in fighting bacterial infections. The *S. aromaticum* and *C. sativum* hexane extracts turned out to be the extracts that displayed AQS potential. These two extracts were able to hinder quorum sensing in CV12472, via the inhibition of violacein production.
Future work can allow for the exact determination of compounds involved in the AQS activity, using gas chromatography (GC-MS). Molecular docking can also be used to speculate of the exact mode of action of bioactive compounds in both hexane extracts of clove and coriander.

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CHAPTER 4 - ANTIVIRULENCE ACTIVITIES OF ACTIVE EXTRACTS OF *SYZYGIUM AROMATICUM* AND *CORIANDRUM SATIVUM* AGAINST *PSEUDOMONAS AERUGINOSA*

4.1. Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that is very common in nosocomial infections. This pathogen rarely infects healthy hosts however can severely affect patients infected with HIV, cystic fibrosis as well as patients with cancer (Bassetti *et al.*, 2018). *P. aeruginosa* is also part of multidrug-resistant pathogens and the emergence of more multidrug-resistant strains, contributes to the antibiotic treatments becoming a concern due to the increase of misuse or overuse of these antibiotics (Ventola, 2015).

Additionally, this pathogen makes use of plethora of virulence factors to cause disease in their host (Lee & Zhang, 2015). The bacterium makes use of their motility to translocate to the infection target sites, take advantage of the host to utilise all the cofactors available to complete biological processes (Khan *et al.*, 2020). *Pseudomonas aeruginosa* also evades the immune system of the host through secreting specific proteases, such as elastase, that can degrade immunoglobulins. Moreover, because this pathogen is capable of forming biofilms, it becomes difficult to treat and is resistant to antibiotics (Khan *et al.*, 2020).

Although all these factors are crucial for *P. aeruginosa* to cause infections, these factors are not always active. The pathogenicity or virulence is controlled by quorum sensing (QS) and the factors only become active once they are needed. These virulence factors are under the control of four interconnected QS systems in *P. aeruginosa*: *las*, *rhl*, *pqs* and *iqs*. These four QS systems allow the bacterium to cause diseases by expressing genes that encodes elastase, that can degrade elastin tissues (Spencer *et al.*, 2010), rhamnolipids expression is essential for biofilms formations (Pamp & Tolker-Nielsen, 2007), pyocyanin expression causes the formation of

reactive oxygen species (e.g., hydrogen peroxide) that cause tissue damage in the infected host (Hall *et al.*, 2016, Castañeda-Tamez *et al.*, 2018). Motility genes are also expressed in order for the bacterium to be mobile (Marko *et al.*, 2018). This information is valuable as it allows researchers to come up with new ways of preventing *Pseudomonas* infections.

In this chapter, *Coriandrum sativum* (coriander) and *Syzygium aromaticum* (clove) hexane extracts were evaluated to interrupt some QS controlled virulence factors and validate their potential against *Pseudomonas aeruginosa* through the following virulence assays: biofilms formation inhibition, twitching motility, swimming motility, swarming motility, elastase inhibition and pyocyanin inhibition.

4.2. Material and methods

4.2.1. Biofilm formation inhibition

This assay was achieved using the 96-well microtiter plate technique and crystal violet (Coffey & Anderson, 2014). An overnight culture of *P. aeruginosa* ATCC 9721 was standardized to OD_{600nm} of 0.1 and 100 µl of standardized cells was transferred to the wells of the microtiter plate. The cells were mixed with 100 µl of LB broth and incubated for 8 hours, in sealed microtiter plates (Greiner Bio-one, Germany) to allow for biofilms formation in the wells. Following this, extracts (1.00 mg/ml) and controls were added to the wells containing the bacterial incubated cultures. The concentration of 1.00 mg/ml was used to directly compare the potency of the extracts. The negative control contained 100 µl of sterile broth mixed with the standardized cells. Other controls in this experiment included 1% DMSO (Merck, South Africa), 0.01 mg/ml of Ciprofloxacin (Merck, South Africa) in 100 µl of broth. After the addition of extracts and controls, all the samples were incubated for 24 hours at 37ºC.

After incubation, the contents from the microtiter wells were dried in an oven (EcoTherm Labotec, South Africa) at 60ºC for 45 minutes and later incubated at room temperature for 15 minutes. Following the drying process, the unbound cells were washed with ddH_2O three times. 100 µl of 0.10% crystal violet was added to the bound cells inside the rinsed wells. The mixture was incubated for 15 minutes in the dark.

The excess crystal violet (Merck, South Africa) was washed away with ddH_2O and the samples were solubilized using 150 µl of 95% ethanol and exported into new wells. The results were obtained by reading the absorbance of the samples at 585nm. The percentage of inhibition cell attachment inhibition was calculated, as follows, to quantify the inhibition of biofilm formation:

$$Percentage\ inhibition = \frac{OD585\ control\ - OD585\ experimental}{OD585\ control} \times 100$$

Saxena *et al.* (2014) classified bacterial biofilm strength according to their optical density. OD readings falling below 0.062 are considered as non-forming biofilms. Between 0.062 and 0.124, biofilms are considered as weak. Moreover, between 0.124 and 0.248, moderate biofilms form. Lastly, above 0.245, biofilms are considered as strong

4.2.2. Motility assays

The procedure to be followed will come from Al-Haidari *et al.* (2016) with some modifications. For the twitching motility, overnight cultures of *P. aeruginosa* were prepared and adjusted to 0.5 McFarland equivalent to 0.1 at OD_{600nm}. Aliquots of 2.0 µl of *P. aeruginosa* were inoculated, through stab-inoculation, on 1% LB plates with extracts (1.00 mg/ml), and without plant extracts (controls). The samples on the plates were incubated at 37 º C for 24 hours. Following incubation, the twitching diameters were measured in mm using a ruler, at the interface between the agar and plate (Murray *et al.*, 2010).

For swimming motility, aliquots of 2.0 µl of bacterial cells were stab-inoculated, with and without extracts on 0.3 % of swimming media (1% tryptone, 0.5% NaCl, 0.5% agar). The cells were incubated for 24 hours at 37ºC. The diameters of the distance travelled by bacterial cells, from the point of inoculation, were measured using a ruler to determine the presence of swimming motility.

For the swarming motility, swarming media were prepared (0.5% agar, 0.5% peptone, 0.2% yeast extract, and 1.0 % glucose). Aliquots of 2.0 µl of bacterial cells were inoculated on swarming media with and without plant extracts and incubated at

37ºC for 24 hours. Following incubation, the swarming diameters were measured to determine the effects of the herbal extracts on the swarming motility of *P. aeruginosa*.

4.2.3. Elastase inhibition assay

The elastase activity was assessed by following the procedure from Ouyang *et al.* (2016) with modifications. The bacterial cells were grown in Peptone Trypticase Soy Broth (PTSB) medium (17.0 g Tryptone, 3.0 g Soytone, 2.5 g Glucose, 5.0 g NaCl, 2.5 g H_2PO^4) overnight and adjusted to an OD_{600} of 0.1. The cultures were aliquoted into centrifuge tubes in the presence and absence (control) of the different plant extracts (1.00 mg/ml). The contents were centrifuged, following an incubation period of 6 hours at 37ºC, and the supernatant was filtered (nylon filter 0.22 µm, Merck). Aliquots of 100 µl of the filtrates were collected and added to 10 mg/ml of elastin Congo red (Merck, South Africa) and 900 µl of Na_2HPO_4 (pH 7.0). The contents were incubated with shaking (150 rpm) at 37ºC for 2 hours. Following this, the contents were centrifuged at maximum speed for 10 minutes, and the absorbance of the supernatant was read at 495 nm. The control supernatants contained the substrate (elastin Congo red) as well as the elastase isolated from the bacterium, in the other hand, the experimental samples contained the substrate, the elastase protease as well as the active extract. The activity of the extracts on elastase inhibition was determined by calculating the percentage of elastase inhibition.

$$Percentage\ elastase\ inhibtion = \frac{OD495\ control\ - OD495\ experimental}{OD495\ control} \times 100$$

The *Pseudomonas* elastase protease cleaves elastin and releases the Congo red dye that was associated with it. Therefore, this will represent a functional protease (negative result). In contrast, the non-release of the dye will mean that the protease was inhibited (positive result).

4.2.4. Pyocyanin inhibition assay

This assay was performed following the procedure from Al-Haidari *et al.* (2016), Mukherjee *et al.* (2017). Pyocyanin measurements was carried out in triplicates, in a King A broth medium (2% peptone, 1% Potassium sulphate, 0.1% magnesium chloride). Standardized overnight cultures of the pathogen (0.5 McFarland equivalent; 500 µl) was inoculated in 5 ml of King A with and without extracts (200 µl); the contents were incubated for 24 hours at 37ºC. Pyocyanin was extracted by adding 3 ml of Chloroform (Merck, South Africa) and 1 ml of 0.2N of HCl. The absorbance was read using a spectrophotometer at 520 nm (SpectraMax DU720, Beckman Coulter, South Africa). The concentration of pyocyanin (in µg/CFU) was determined by multiplying the OD_{520} values obtained, by 17.072 (Mukherjee *et al.*, 2017), (Essar *et al.*, 1990). The concentrations of pyocyanin were compared between the experimental samples and the control to determine the effects of the active extracts on the amount of pyocyanin inhibited in *P. aeruginosa*.

4.2.5. Thin Layer Chromatography-Bioautography assay

This step consisted of using the dried chromatograms, using the TLC method, that contained compounds separated with Hexane: acetone at 9:1 ratio (Rodriguez *et al.*, 2014). The two bioactive extracts were mixed with 1.00 ml hexane (Merck Millipore, South Africa) and were sonicated. The extracts were later spotted on the silica gel TLC plates (Merck, silica gel 60 F_{254}^{S}, South Africa) that were placed in a TLC chamber containing 9:1 ratio of hexane: acetone as mobile phase. The plates were developed to 5.0 cm.

The procedure to be followed was similar to (Tiwary *et al.*, 2017). The TLC plates were placed in LB plates that were overlaid with an overnight culture of *P. aeruginosa* grown and contained in 0.3% agar. The samples were incubated for 24 hours at 37ºC, and the results were analysed. After incubation, the plates were sprayed with the INT redox indicator and incubated again for 1 hour at 37ºC. Observations were made following the incubation. The zones where the bacterium would grow appeared red (as a result of the redox indicator). The absence of the red

colour indicated inhibition of the bacterium growth. Additionally, the separated compounds should form spots that will be used to calculate the retention factor (Rf) of the compounds. The Retention factor (Rf) values of spots were determined by using the equation below and compared to reference *Rf* zones from plant compounds that are known.

$$Rf = \frac{distance\ run\ by\ the\ solute/compound}{distance\ run\ by\ the\ solvent}$$

4.2.6. Chemical profiling of the active extracts

Gas-chromatography Mass spectrometry analysis was carried on a Shimadzu QP2100 SE (Shimadzu Corporation, Tokyo, Japan) with an inertCap 5 MS/NP capillary column (30 m x 0.25 mm x 0.25 µm: GL Sciences, Tokyo, Japan). A volume of 1.00 µl of each active extract sample was injected into the GC-MS. The carrier gas, Helium, was used at a constant flow rate of 1.0 ml/min at 11°C min^{-1}, and held at this temperature for 14 min. The analysis was carried out at 70eV in the electron impact ionization mode. Compounds were compared against published mass spectra libraries NIST 11.

4.2.7. Statistical analysis

Results are presented as a standard deviation that was calculated from the mean values calculated in GraphPad Prism version 8.4.3. One way ANOVA was also used to determine the significance of the assay results. Statistical difference is considered significant if $p<0.05$.

4.3. Results and discussion

4.3.1. Biofilm inhibition

P. aeruginosa is an important pathogen relying on its virulence factors to cause diseases. In addition to that, the bacterium forms biofilms that grant additional protection against drugs. This behaviour is also responsible for their increased resistance against these drugs. Degradation of biofilms is often considered as a gold standard when it comes to antiquorum sensing studies (Annapoorani *et al.*, 2013). Here, we assessed the potential of the *S. aromaticum* (clove) and *C. sativum* (coriander) hexane extracts against the ability of *P. aeruginosa* to form biofilms in *in vitro*.

The results **(figure 4.1)** demonstrated that *P. aeruginosa* was able to form biofilms in the presence of the active extracts. However, the biofilms produced were significantly lower (*$p<0.05$) compared to the culture control as well as the positive control (ciprofloxacin). The clove and coriander hexane extracts, at 1.00 mg/ml, reduced the biofilm cell attachment by 70.3% and 80.1% respectively, which is significantly higher than the inhibition of cell attachment by ciprofloxacin (30.2%). Based on the classification by Saxena *et al.* (2014), *P. aeruginosa* formed moderate biofilms in the presence of hexane extracts of *C. sativum* and *S. aromaticum* at 1.00 mg/ml. This result highlights the great potential these two extracts have in fighting bacterial infections by interfering with *Pseudomonas* biofilms development through antiquorum sensing.

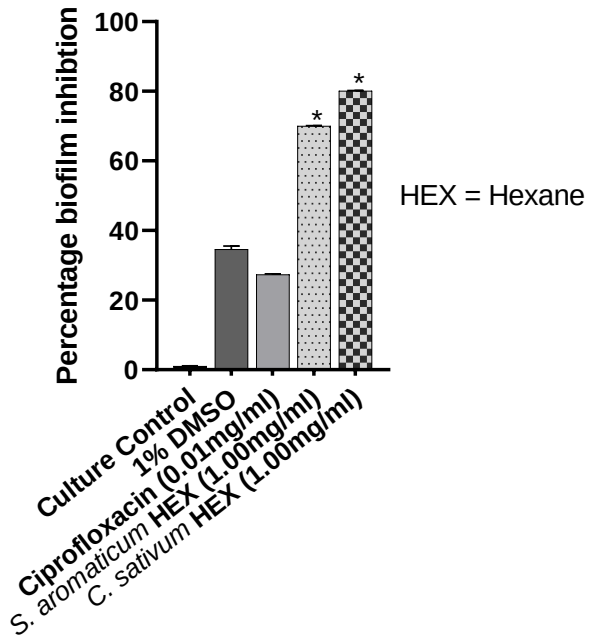

Figure 4.1. S. aromaticum* and *C. sativum* hexane extracts reduce the biofilms formation of *P. aeruginosa (*p<0.05)

Both *S. aromaticum* and *C. sativum* hexane extracts were able to interfere with the ability of *P. aeruginosa* to form biofilms*. S. aromaticum* is popular in antibiofilm studies. Abdul Majeed (2018) demonstrated the effectiveness of the *S. aromaticum* extracts against some oral pathogen's biofilms, including *P. aeruginosa* in which the aqueous and ethanol extracts managed to reduce biofilms by 49% and 65.3%, respectively. The researcher highlighted the importance of these extracts into fighting against bacterial infections as well as being used as a preventive method. Rathinam & Viswanathan (2018) also displayed and highlighted the importance and ability of *S. aromaticum* extracts to disrupt *Pseudomonas* biofilms with the highest inhibition at 59.98% at a concentration of 700 µg/ml.

Concerning the *C. sativum* extracts, Al-Haidari *et al.* (2016) demonstrated that methanolic extracts of coriander significantly affected biofilms in *Pseudomonas aeruginosa* PA14. Molina *et al.* (2020) also confirm the potential of coriander in eliminating biofilms. The hexane extracts of coriander they use inhibited *P. aeruginosa*

PAO1 biofilms by almost 30% at just 0.10 mg/ml. Molina *et al.* (2020) identified oleic acid as the main constituent of their bioactive extracts. Mirani *et al.* (2017) elucidated that this compound could interact with the hydrophobic cell membrane of *S. aureus* and consequently preventing the formation of biofilms. The systems involved in biofilm formation in *P. aeruginosa* are *las* and *rhl* (Fila et *al*., 2018). This experiment suggests that the clove and coriander extracts have an inhibitory effect on either *las*, *rhl* or both systems simultaneously either by competitive binding to signal receptors or degradation of the signal molecules.

4.3.2. Motility assays

Another virulence factor under QS is motility. Motility in *P. aeruginosa* is an important virulence factor as the bacteria will need to translocate to favourable environments, once inside the host. The bacterium can move through the solid medium using twitching motility mediated by the type IV pili (Mattingly *et al.*, 2018). For this reason, the clove and coriander extracts were used to determine their effects on *Pseudomonas aeruginosa* motility. All observations were recorded by measuring the diameters of twitching zones, swimming zones and swarming zones and graphs were generated from these measurements (**figures 4.2, 4.3 and 4.4**).

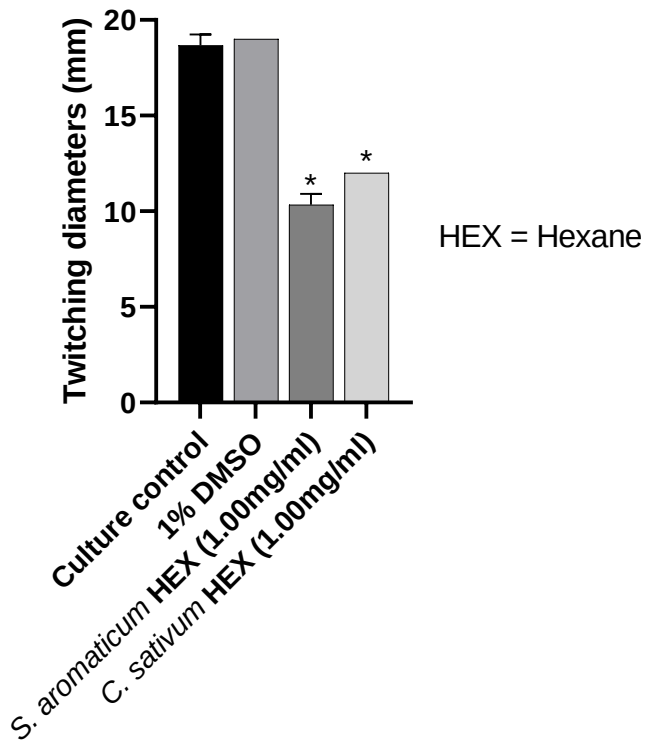

Figure 4.2. S. aromaticum* and *C. sativum* hexane extracts reduce the twitching diameters in *Pseudomonas aeruginosa (*p<0.05)

The culture control, as well as the 1% DMSO control, did not differ with diameters of 18.66 mm and 19.00 mm, respectively (**figure 4.2**). On the other hand, the two selected extract samples showed to have reduced the twitching diameters with 10.66mm (43% reduction) for *S. aromaticum*, and 12.00 mm (36% reduction) for the *C. sativum* extract. The extracts, therefore, have the potential ability to interfere with the twitching motility in *Pseudomonas aeruginosa*, at a concentration of 1.00 mg/ml.

Twitching in *P. aeruginosa* is mediated by the type IV pilus (TFP), a polar filamentous appendage, that allows the bacterium to translocate on solid surfaces. The flagella and TFP promote cell attachment to the host and the sensitivity of the bacteria to the surface. Upon binding to a surface, the bacteria upregulate LasR (Response regulator in the *las* system) to activate virulence genes by responding to its ligand PA-I (3OC12-HSL), the *las* autoinducer (Chuang *et al.*, 2019). The inhibition observed by both coriander and clove on twitching motility can be explained by the presence of a compound or a mixture of compounds that are able to interfere with the upregulation of LasR, consequently lowering the factors needed to perform twitching

motility. Without this type of twitching translocation, the pathogen will be unable to form microcolonies that normally develop prior to biofilms becoming mature (Unosson, 2015).

Concerning swimming motility, both the *S. aromaticum* (clove) and *C. sativum* (coriander) hexane extracts reduced the swimming motility of *P. aeruginosa* by 16.00% (16.00 mm), compared to the culture control (19.00 mm) (**figure 4.3**). However, this reduction is not significant (ns - p>0.05), and both samples are not significantly effective against the swimming motility of *P. aeruginosa*.

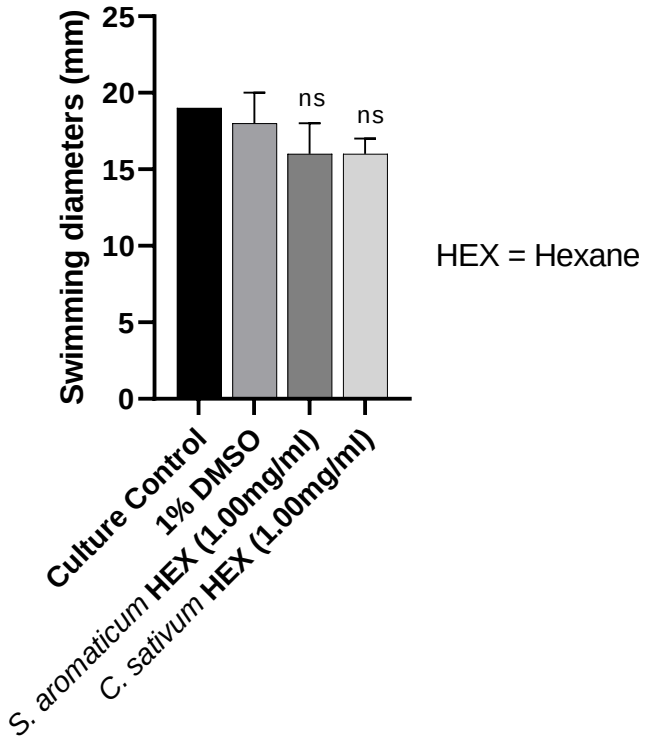

Figure 4.3. S. aromaticum* and *C. sativum* hexane extracts do not have any effects on the swimming motility of *Pseudomonas aeruginosa (p>0.05: not significant - ns)

Swimming motility refers to the movement of *P. aeruginosa* in a liquid or low-viscosity medium. The bacterium makes use of a polar flagellum that rotates clockwise or counter clockwise to propel the bacterium towards or away from a signal. This type of motility only requires the flagellum to be operational and therefore no quorum sensing is involved in this specific type of motility (Ha *et al.*, 2014).

Looking into swarming motility, only *C. sativum* hexane extract sample had a significant decrease (35%; 13.00 mm) in the swarming motility of *P. aeruginosa* compared to the control (20.00 mm) and the *S. aromaticum* sample (10%; 18.00 mm) (**figure 4.4**).

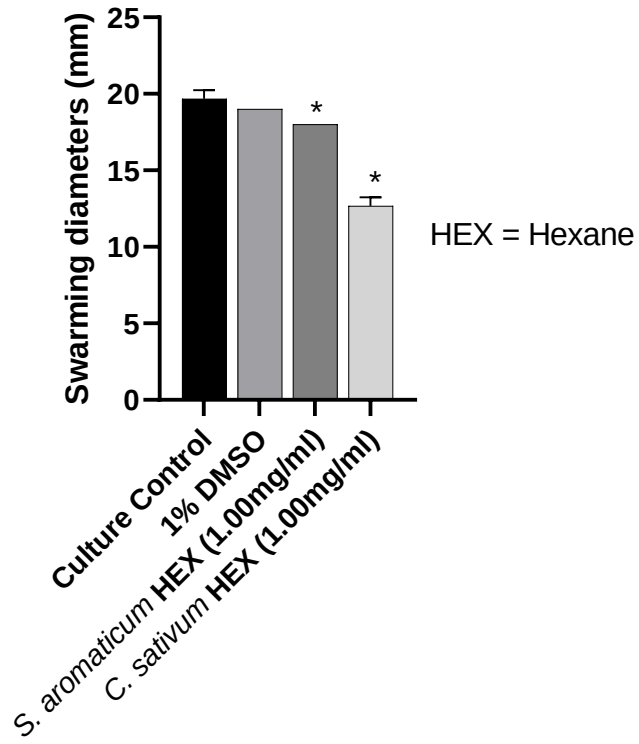

Figure 4.4. *C. sativum* hexane extracts reduce the swarming motility in *Pseudomonas aeruginosa* whereas *S. aromaticum* has no effect. (*p<0.05)

Swarming motility refers to the movement of the bacterium on semi-solid surfaces. This motion is achieved by making use of both the flagella and type IV pili (Fraser & Hughes, 1999). Unlike swimming motility, swarming motility requires an additional production of biosurfactants such as rhamnolipids, to reduce surface tension (Oura *et al.*, 2015). Such biosurfactant production is regulated via the *rhl* system in *P. aeruginosa*. This experiment displays that coriander is able to interfere with the *rhl* system in *P. aeruginosa* via competitive binding to the RhlR regulator or degradation of the C4HSL signalling molecule.

P. aeruginosa employs a plethora of mechanisms to invade and infect the host successfully. One important mechanism is motility which is also linked to quorum

sensing. The bacterium needs to be motile to reach the target organs in the host. By activating its *las* and *rhl* systems, the bacterium can successfully infect a new host. Despite this, *C. sativum* showed some evidence of interfering with the twitching and swarming motility, although the differences were small, especially for the swarming motility. *S. aromaticum* displayed a potential AQS against twitching motility only. These observations highlight the complexity of motility and its associated molecules and interactions (Overhage *et al.*, 2008) but also the potential of *C. sativum* & *S. aromaticum* in interfering with the motility of *P. aeruginosa*, with motility being an important step in infections as it normally precedes the cell attachment and the formation of biofilms.

4.3.3. Elastase inhibition assay

Elastase is a multifunctional metalloprotease from *P. aeruginosa* and is encoded by the *las* system and regulated through QS (Galdino *et al.*, 2019). This protease degrades the extracellular matrix constituents of the host cells, such as elastin, which is needed to maintain the elasticity of the lungs and blood vessels (Li *et al.*, 2019). Elastase also degrades several components of the host immune system such as macrophages and Immunoglobulins, allowing to escape the immune response (Kuang *et al.*, 2011).

Based on the findings, the AQS potential of *S. aromaticum* and *C. sativum* were tested for their ability to inhibit the elastase protease in *P. aeruginosa*. This was achieved using the substrate elastin Congo red.

From the results obtained in **figure 4.5**, *C. sativum* (coriander), at 1.00 mg/ml, was unable to inhibit the protease as the dye was able to be released in the sample, in comparison to the control sample.

On the other hand, *S. aromaticum* (clove), at 1.00 mg/ml, was able to inhibit the protease. The elastin-Congo red complex was not cleaved, and therefore, the dye was not released in the sample. This activity represented about 59% reduction in the elastase activity in comparison to the control sample.

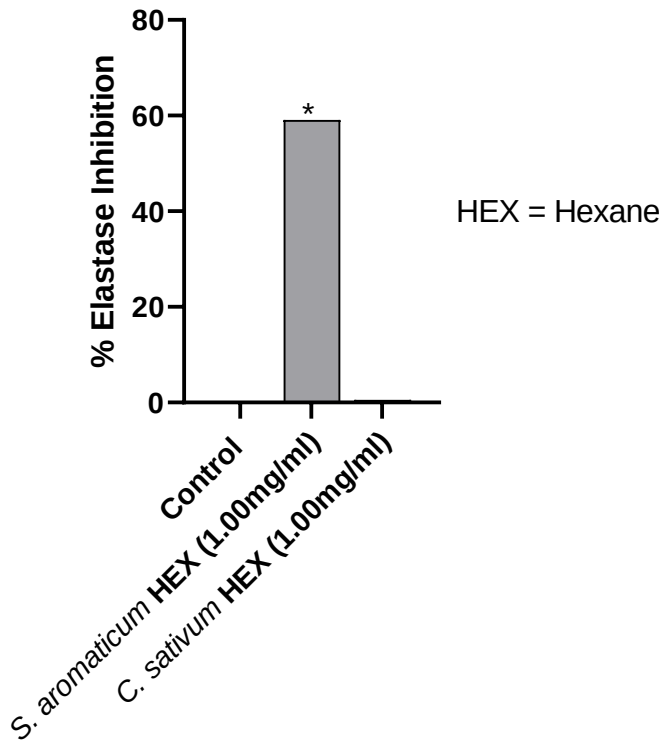

Figure 4.5*. S. aromaticum* inhibits the elastase from *Pseudomonas aeruginosa* whereas the *C. sativum* has no effect on elastase (*p<0.05).

The activity observed with the clove extract gives the information that the clove hexane extract can interfere with the *las* system in *P. aeruginosa.* Zhou *et al.* (2012) observed a decrease in elastase activity by up to 46%. By modifying *E. coli* MG4/pKDT17 to produce LasR, by fusing the *lasB* promoter into *lacZ*, they determined that the elastase inhibition observed was due to the binding of eugenol to the LasR receptor, consequently reducing the synthesis of virulence genes under the *las* system. Rathinam & Viswanathan (2018) also demonstrated that clove inhibits the elastase production in *P. aeruginosa* PAO1 by 58.61% and *P. aeruginosa* RRLP1 by 40.38% at 0.70 mg/ml, and *P. aeruginosa* RRLP2 by 60.19% at 0.50 mg/ml. These findings are in correlation with our experiment in which clove was able to inhibit elastase production in *P. aeruginosa* by 59%, enhancing the belief that clove contains compounds or a compound that significantly interfere with the AHL *N*-3-oxo-dodecanoyl homoserine lactone (3OC12-HSL) either by inhibiting binding to its target receptor LasR, degrading the signal or interfering with its synthesis, consequently inhibiting the *las* system in *P. aeruginosa*, and reducing the elastase production in this

bacterium. However, the exact underlaying mechanisms for this inhibition must be explored and further elucidated.

In the case of *C. sativum* (coriander), no inhibition of elastase was observed. This would mean that the hexane extract of coriander is not interfering with the AHL *N*-3-oxo-dodecanoyl homoserine lactone (3OC12-HSL), consequently not interfering with the *las* system in *Pseudomonas aeruginosa*. Furthermore, no studies demonstrate that coriander can inhibit elastase, which reinforces the observation that compounds inside the coriander hexane extracts are mostly effective on the *rhl* system, by inhibiting, preventing the synthesis or binding of butyryl-HSL, the C4 AHL molecule for the *rhl* system (Ghosh *et al.*, 2019).

4.3.4. Pyocyanin inhibition assay

Pyocyanin is a critical virulence factor for *Pseudomonas aeruginosa*, particularly in cytotoxicity during infections (Lee & Zhang, 2015). Pyocyanin is a blue redox compound that allows the bacterium to form reactive oxygen species that lead to the accumulation of hydrogen peroxide, an agent well known to damage cells (Castañeda-Tamez *et al.*, 2018).

In the present study, the extracts were tested for their ability to affect the production of pyocyanin in *Pseudomonas aeruginosa*. From the graph in **figure 4.6**, the control produced pyocyanin at a concentration of 6.83 µg/CFU, the samples incubated with coriander extracts produced 6.32 µg/CFU, representing a reduction of 8% in pyocyanin production, which is considered low and not significant. On the other hand, the pyocyanin production was lower in the samples containing the clove extracts. The concentration of pyocyanin was 4.78 µg/CFU (, which represented a 30% reduction. However, this reduction is not significant ($p > 0.05$). Moreover, lower pyocyanin production was observed in the samples containing the clove extracts. The concentration of pyocyanin was 4.78 µg/CFU, which represented a 30% reduction. However, this reduction is not significant according to the one-way ANOVA performed.

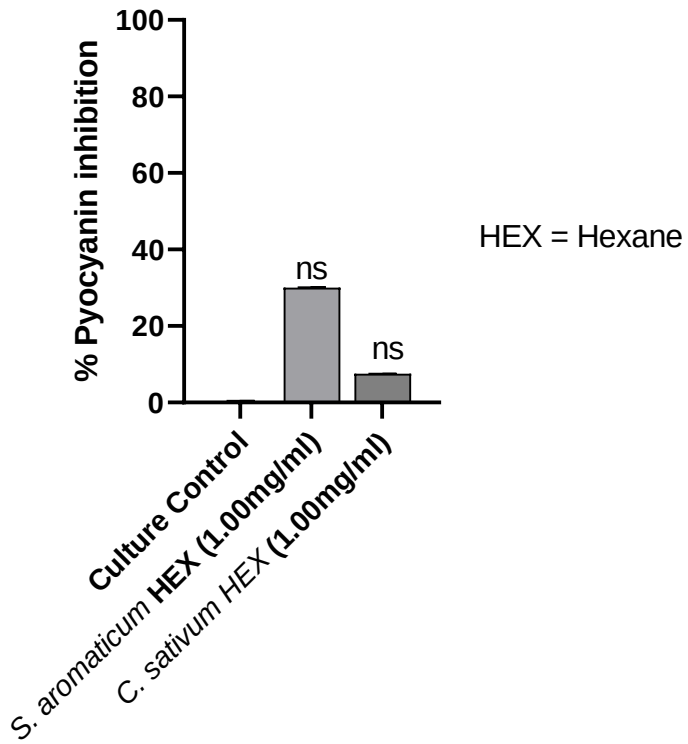

Figure 4.6. *S. aromaticum* or *C. sativum* inhibit the pyocyanin production from *Pseudomonas aeruginosa* (p>0.05 – ns)

Al-Haidari *et al.* (2016) demonstrated that *C. sativum* (coriander) ethanolic extracts reduced the pyocyanin inhibition in their study in which pyocyanin production was reduced to about 2.00 µg/ml. Interestingly, in this current study, the coriander extract did not inhibit pyocyanin production. This observation could be potentially due to the difference in the solvent chosen to extract the compounds. Ethanol and hexane extract different types of molecules. Ethanol extract molecules that are polar whereas hexane extracts non-polar molecules. Another reason could be that *P. aeruginosa* quorum-sensing system is composed of many different systems, with the master regulator being *las (*Lee & Zhang*, 2015)*. The inhibition of *pqs* or *rhl* will not lead to the complete shutdown of quorum sensing, taking that into account along with the fact that pyocyanin production is mainly associated with the *pqs* and *rhl* (Zhou *et al*., 2012), this could explain why no pyocyanin inhibition was observed in this study as this system can also be upregulated by the *iqs* system (Lee & Zhang, 2015).

Additionally, *S. aromaticum*, the clove extract managed to inhibit the pyocyanin production in *Pseudomonas aeruginosa,* however, this reduction was not significant

(p>0.05). The *las* system is the primary regulator of *pqs* and *rhl* (Lee & Zhang, 2015*)*, appeared to have been inhibited, consequently lowering the activity of *pqs* and *rhl*, leading to less virulence in the bacterium. However, this is not accurate in this case. Neither clove or coriander hexane extracts were effective in inhibiting, significantly, the pyocyanin in *Pseudomonas aeruginosa*.

4.3.5. TLC Bioautography of active extracts

The bioautography was performed by developing the extract samples in a thin layer chromatography (TLC) (**figure 4.7**). For *Syzygium aromaticum*, bands could not evident for both extracts during the separation step. The different compounds did not seem to dissociate from the mixture. Additionally, *S. aromaticum* extracts showed a zone of bacterial inhibition (Rf value = 0.64).

In the other hand, the separation from *C. sativum* extracts was not complete, and one green compound and an orange/brown compound were observed. This indicated the presence of 2 main compounds in large amounts. Relating this observation to literature, these two compounds could potentially correspond to E-2-decenal and Linalool. At that site, there was a slight reduction in *Pseudomonas aeruginosa* bacterial growth. This zone of inhibition was located at a distance of 29 mm, corresponding to an R_f value of 0.58.

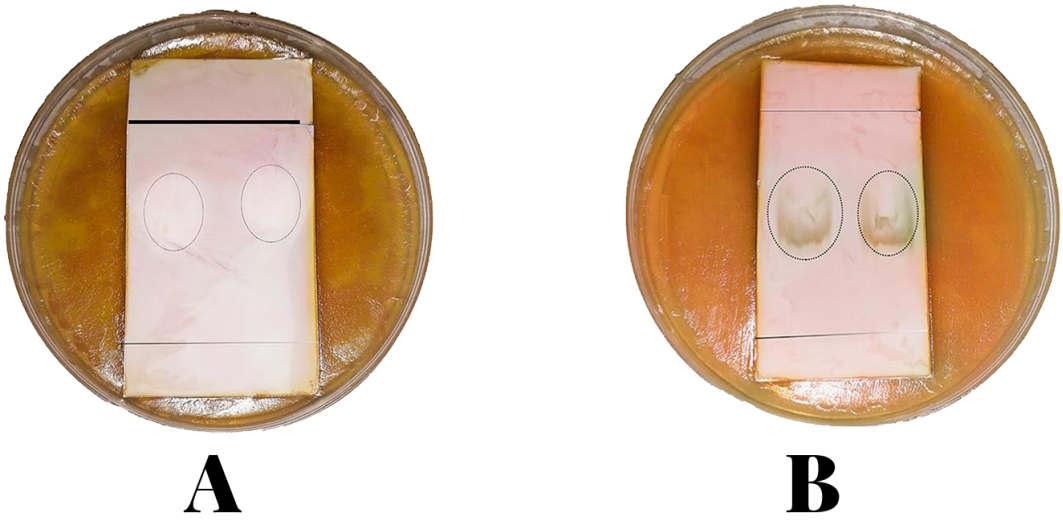

Figure 4.7. TLC bioautography of (A) *S. aromaticum* hexane extract and (B) *C. sativum* hexane extract. (A) No specific band could be seen but only a zone of inhibition was observed from 32mm (circled). The Rf value calculated from this result was 0,64. **(B)** Two major bands can be seen from 29mm (circled). The Rf value calculated is 0.58

Rodriguez *et al.* (2014) separated the essential oils in *S. aromaticum* in their research using the same solvent system (hexane: acetone of 9:1 ratio). They have identified eugenol as the main compound in their *S. aromaticum* sample. This compound had an Rf value of 0.65 in the thin layer chromatography. This value is very close to the value obtained in our experiment (Rf = 0.64). This is information backs up the data obtained from this experiment highlighting the abundance of eugenol in *S. aromaticum* samples. This also confirms again the ability of this compound to be useful in fighting bacterial infections through antiquorum sensing as eugenol is a well-documented AQS compound (Lou *et al.*, 2019). Most studies focus on essential oils of clove which contain concentrated amounts of compounds, instead of extracts that contain a fraction of these concentrated compounds, depending on the solvent. However, all studies detect the same composition for major compounds.

According to Nassar *et al.* (2007), *S. aromaticum* hexane extracts contained phenylpropanoids such as eugenol in a large majority (71.56%) and eugenol acetate (8.99%) as the two major compounds identified. Sesquiterpenes such as caryophyllene oxide was detected at about 1.67%. Other compounds including

monoterpenes, aldehydes, alcohols and ketones were present at concentrations of 1% or less. This study from Nassar *et al.* (2007) is also backed up by another recent study to identify compounds in *S. aromaticum*. Rodríguez *et al.* (2018), as well as Amelia et al. (2017), identified the same major compounds in their research, namely eugenol, eugenol acetate in the relative abundance.

Eugenol is well established as an inhibitor of quorum sensing. It is used in perfumes, as a flavouring agent as well as antiseptics and anaesthesia (Jadhav *et al.*, 2004). Kim *et al.* (2016) determined the mechanism of action of eugenol. They determined that the hydroxyl group, the methoxy group, and the alkyl chain were responsible for the eugenol activity in virulence inhibition. In addition to this, Rathinam *et al.* (2017) determined that the inhibition of quorum sensing in *P. aeruginosa*, by eugenol, was due to the competitive binding of eugenol to the signal receptors, more precisely LasR of the *las* system, which is the master regulator of quorum sensing in *P. aeruginosa*. This type of mechanism is likely responsible for the observations from the virulence assays performed prior to this section. The competitive binding mechanism is due to the structural similarities between eugenol and the acyl-homoserine lactone signals. In *P. aeruginosa*, the main autoinducer signals are 3-oxo-C12-homoserine lactone that activates the *las* system and C4-homoserine lactone, which activates the *rhl* system. All 3 molecules share similarities; an acyl group and carbon side chains.

Regarding the *C. sativum* sample, the Rf obtained for both the brown and green compounds was 0.58. Additionally, the inhibition zone was less than that of the *S. aromaticum* sample. Literature focuses more on *C. sativum* seeds than leaves, although the compound composition is still the same, only the amount of Linalool and E-2-decenal varies. In *C. sativum* leaves, the dominating compound is E-2-Decenal (Mandal & Mandal, 2015) whereas, in *C. sativum* seeds, Linalool dominates (Mandal & Mandal, 2015, Beyzi *et al.*, 2017). The Rf value represents two compounds close to one another with the impossibility to determine their nature. Based on the knowledge of the coriander leaves composition, the compounds may correspond to Linalool and E-2-Decenal, but it is unclear as it needs more investigations.

Shahwar *et al.* (2012) characterised the compound composition in *C. sativum* seeds and leaves. They have identified several major compounds in their leaf samples. The main compounds were (E)-2-decenal at 32.23% and Linalool at 13.97%. They also identified several other compounds in smaller quantities. Those compounds were (E)-2-dodecenal at 7.51%, (E)-2-tetradecenal at 6.56%, 2-decen-1-ol at 5.45%, (E)-2-undecenal at 4.31%, dodecanal at 4.07%, (E)-2-tridecenal at 3.00%, (E)-2-hexadecenal at 2.94%, (E)-2-pentadecenal at 2.47% and finally α-pinene at 1.9%.

This compound composition is similar to a study by Mandal & Mandal (2015) where they also obtained a similar composition of their *C. sativum* leaves from Brazil. This composition is also similar in a more recent study by Chahal *et al.* (2017) in which the major compounds in *C. sativum* were E-2-Decenal and Linalool.

E-2-Decenal is an analogue of cinnamaldehyde in which the aromatic ring has been replaced with an alkyl group. Brackman *et al.* (2011) demonstrated that this analogue could inhibit the AI-2 signal molecule in *Vibrio* spp by 57% (50 µM) and 90% (100 µM), thus reducing *Vibrio spp* bioluminescence, which is regulated by a QS system. Moreover, Brackman *et al.* (2011) determined that the mode of action of cinnamaldehyde analogues, in their study, was the binding of these analogues to LuxR, preventing the binding of this LuxR regulator to its target DNA that activates the quorum sensing. This mechanism was also reviewed by Doyle & Stephens (2019). In *CV12472*, the CviI and CviR are homologs of LuxI/LuxR, therefore the inhibition of violacein by *C. sativum* hexane extract in the antiquorum sensing tests is due to E-2-decenal binding to CviR and preventing the activation of *vio* genes, and consequently blocking quorum sensing. In *P. aeruginosa*, the LuxR types of receptors are both LasR and RhlR, indicating that E-2-decenal can also affect *Pseudomonas aeruginosa* in the same fashion.

4.3.6. Chemical profiling of active extracts

Chemical profiling was performed for the active extracts using the Gas Chromatography technique (GC-MS). This technique allows for the detection of chemical compounds within a mixture over time.

The chemical profile of both *S. aromaticum* and *C. sativum* hexane extracts are shown in **figure 4.8 and 4.9**. The compounds present belong to different chemical classes of fatty acids, sugars and ketones. The full details are presented in **table 4.1**

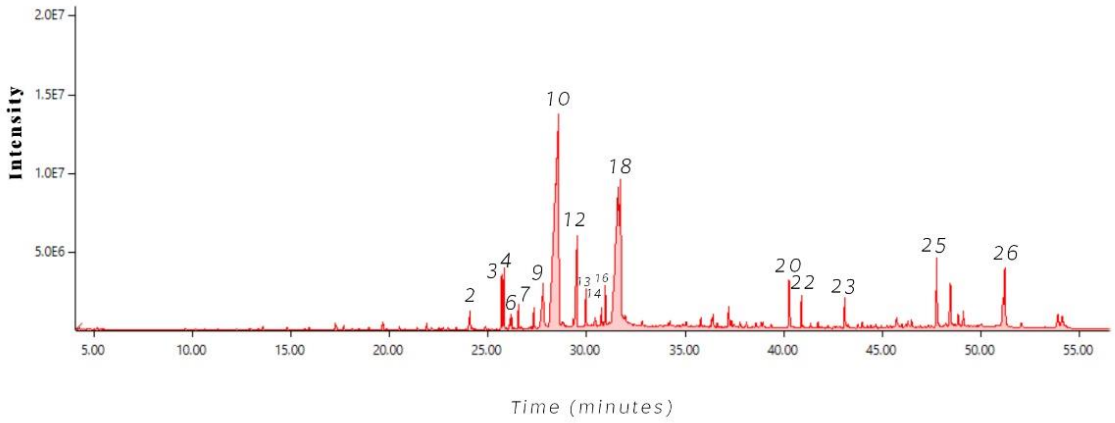

Figure 4.8. Representative total ion chromatograph (TIC) of *Syzygium aromaticum* (Hexane) extracts. Peaks correspond to the data presented in Table 4.1

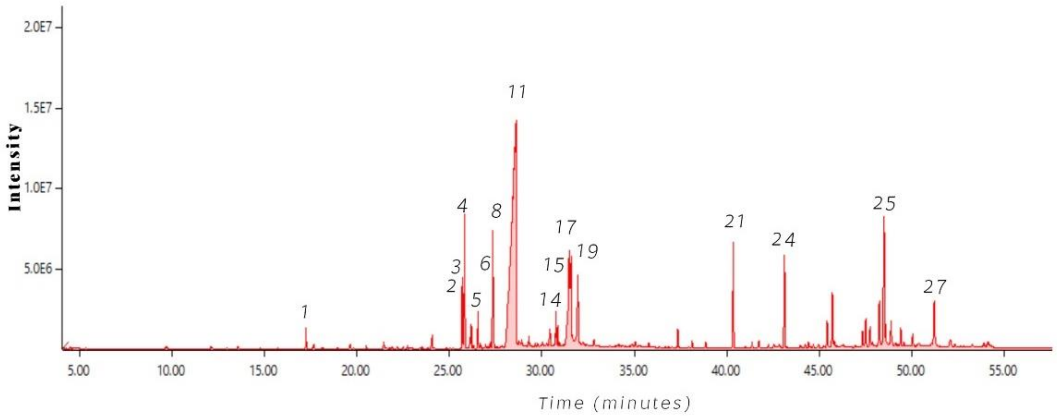

Figure 4.9. Representative total ion chromatograph (TIC) of *Coriandrum sativum* (Hexane) extracts. Peaks correspond to the data presented in Table 4.1

Table 2.1. GCMS spectral analysis of *Coriandrum sativum* and *Syzygium aromaticum* hexane extracts

Peak #	Rt	Name	Molecular weight	Molecular formula	Hexane extracts	
					C. sativum	*S. aromaticum*
1	17.250	2,6,10-Trimethyldecane	226.4	$C_{16}H_{34}$		0.8%
2	24.094	Tetradecanoic acid	228.4	$C_{14}H_{28}O_2$	1.2%	
3	25.689	Neophytadiene	278.5	$C_{20}H_{38}$	2.0%	1.5%
4	25.828	2-Pentadecanone, 6,10,14-trimethyl-	268.5	$C_{18}H_{36}O$	3.8%	4.9%
5	26.187	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	296.5	$C_{20}H_{40}O$		0.4%
6	26.545	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	296.5	$C_{20}H_{40}O$	0.8%	0.7%
7	27.319	2-Piperidinone, N-[4-bromo-n-butyl]-	234.1	$C_9H_{16}BrNO$	0.8%	
8	27.348	Phytol	296.5	$C_{20}H_{40}O$		2.5%
9	27.796	7,10,13-Hexadecatrienoic acid, (Z,Z,Z)-	250.4	$C_{16}H_{26}O_2$	5.7%	
10	28.559	Butanoic acid	88.1	$C_4H_8O_2$	41.1%	
11	28.582	n-Hexadecanoic acid	256.4	$C_{16}H_{32}O_2$		26.8%
12	29.514	3,4-2H-Isocoumarin-3-one, 4,4,5,7,8-pentamethyl-	232.3	$C_{14}H_{16}O_3$	4.5%	
13	29.965	4-Hydroxy-5,7-dimethoxyquinoline-3-carbonitrile	230.2	$C_{12}H_{10}N_2O_3$	2.0%	
14	30.759	2(3H)-Furanone, 5-heptyldihydro-	184.3	$C_{11}H_{20}O_2$	2.0%	2.2%
15	30.850	1-Hexadecyn-3-ol, 3,7,11,15-tetramethyl-	294.5	$C_{20}H_{38}O$		0.6%
16	30.943	Phytol	296.5	$C_{20}H_{40}O$	3.1%	
17	31.473	13-Octadecenal, (Z)-	266.4	$C_{18}H_{34}O$		2.0%
18	31.604	Linolelaidic acid	280.4	$C_{18}H_{32}O_2$	23.7%	
19	31.947	Octadecanoic acid	284.5	$C_{18}H_{36}O2$		1.8%
20	40.261	n-Tetracosanol-1	354.7	$C_{24}H_{50}O$	2.4%	
21	40.347	Heptacosane	380.7	$C_{27}H_{56}$		4.8%
22	40.888	Erucic acid	338.6	$C_{22}H_{42}O_2$	1.4%	
23	43.087	Octacosanol	410.8	$C_{28}H_{58}O$	1.6%	
24	43.114	Hentriacontane	436.8	$C_{31}H_{64}$		4.4%
25	48.491	γ-Sitosterol	414.7	$C_{29}H_{50}O$	1.3%	2.0%
26	51.205	3,7,11,15-Tetramethylhexadec-2-en-1-yl acetate	338.5	$C_{22}H_{42}O_2$	2.5%	
27	51.208	18-Nonadecen-1-ol	282.5	$C_{19}H_{38}O$		1.0%

The compounds present in the extracts, according to the GCMS, belong to different classes of chemicals such as fatty acids, steroids, terpenes, alkenes, ketones, lactams, carboxylic acids and furanones.

At first sight, the compound composition from both *C. sativum* (coriander) and *S. aromaticum* (clove) in this experiment do not contain major compounds that are usually found. These compounds are Eugenol (Rodríguez *et al.*, 2018) for clove as well Linalool and E-2-Decenal for coriander (Mandal & Mandal, 2015). The absence of these compounds is likely due to the loss of active compounds over time of the study, especially the important volatiles. However, the GCMS still yielded various compounds that can be of interest.

Both *C. sativum* and *S. aromaticum* have some compounds in common. These compounds are **(3)** Neophytadiene, **(4)** 2-Pentadecanone, 6,10,14-trimethyl-, **(6)** 3,7,11,15-Tetramethyl-2-hexadecen-1-ol, **(8,16)** Phytol, **(14)** 2(3H)-Furanone, 5-heptyldihydro- and **(25)** γ-Sitosterol.

Neophytadiene and Phytol can be potentially AQS compounds based on molecular docking scores demonstrated by Baloyi *et al.* (2021). Neophytadiene and Phytol displayed weak binding affinities to the CviR receptor of *C. violaceum*. In the other hand Phytol had an improved score in binding affinity to the protein 2VU0, in *P. aeruginosa*, that is activated by the C12-HSL molecule (Baloyi *et al.*, 2021). The 2(3H)-Furanone, 5-heptyldihydro- compound could also be of interest. This compound shares structural similarities to AHL molecules and may possess antivirulence activity against *P. aeruginosa* quorum sensing systems. Moreover, there are no known antivirulence activity from 2-pentadecanone, 6,10,14-trimethyl-, 3,7,11,15-Tetramethyl-2-hexadecen-1-ol and γ-Sitosterol. These compounds will still need to be tested.

Furthermore, *C. sativum* hexane extract contains unique compounds that are in not found in *S. aromaticum*. These compounds are **(2)** Tetradecanoic acid, **(7)** 2-Piperidinone, N-[4-bromo-n-butyl]-, **(10)** Butanoic acid, **(12)** 3,4-2H-Isocoumarin-3-one, 4,4,5,7,8-pentamethyl-, **(13)** 4-Hydroxy-5,7-dimethoxyquinoline-3-carbonitrile, **(18)** Linoelaidic acid, **(20)** n-Tetracosanol-1, **(22)** Erucic acid, **(23)** Octacosanol and **(26)** 3,7,11,15-Tetramethylhexadec-2-en-1-yl acetate. The major compounds

identified in *C. sativum* were **(10)** Butanoic acid and **(18)** Linoelaidic acid with 41.1% and 23.7% respectively.

Butanoic acid or butyric acid has no proven properties in interfering with the virulence of *P. aeruginosa*, therefore it is not a compound of interest in this case despite its high presence in *C. sativum* hexane extracts. Additionally, Linoelaidic acid or linoleic acid (LA) is a polysaturated fatty acid in coriander and the second highest major compound as per the GCMS results. LA possesses antivirulence effects on *P. aeruginosa* PA14 by inhibiting the propagation of biofilms in in this bacterium through binding to the receptors of diffusible signal factors (DSF) (Kim *et al.*, 2021). The effect of LA on *Pseudomonas* biofilms was also displayed by Chanda *et al.* (2017) making LA a good compound to tackle *Pseudomonas* infections through interference with quorum sensing.

Tetradecanoic acid is known to possess antivirulence properties against *P. aeruginosa* PA14. According to Juárez-Rodríguez *et al.* (2021), Tetradecanoic acid is able to reduce pyocyanin production by 58% at 1,000 µM which displays this compound's ability to interfere with *P. aeruginosa* quorum sensing systems. Further, the rest of the compounds unique to coriander do not have any documented activity in interfering with the quorum sensing of *P. aeruginosa* leaving a gap for further investigation in finding if these compounds can be tested to find any antiquorum sensing property.

In the other hand, the *S. aromaticum* hexane extract also contained unique compounds such as **(1)** 2,6,10-Trimethyldecane, **(11)** n-Hexadecanoic acid, **(15)** 1-Hexadecyn-3-ol, 3,7,11,15-tetramethyl-, **(17)** 13-Octadecenal, (Z)-, **(19)** Octadecanoic acid, **(21)** Heptacosane, **(24)** Hentriacontane and **(27)** 18-Nonadecen-1-ol. The major compound in this extract was **(11)** n-Hexadecanoic acid at 26.8%. N-Hexadecanoic acid has no recorded activity against *P. aeruginosa* and further studies is needed to assess if this compound can affect the QSS of the bacterium. Additionally, all other compounds identified in the GCMS procedure for *S. aromaticum* do not have any recorded AQS activity against *P. aeruginosa*.

These results display that both *C. sativum* and *S. aromaticum* possess diverse types of compounds making them ideal for researching new candidates to combat infections.

However, these diverse compounds must be studied further to possibly detect desirable AQS properties.

4.4. Conclusion

Not only the two hexane extracts of *Syzygium aromaticum* (clove) and *Coriandrum sativum* (coriander) could interfere with violacein production in CV12472, they also displayed promising potential against *Pseudomonas aeruginosa* virulence factors through AQS. Both extracts displayed good inhibition of *Pseudomonas* biofilm cell attachment as well as the twitching motility of the pathogen. On the other hand, the clove sample was a good performer against these virulence factors as it also inhibited elastase in *Pseudomonas aeruginosa*. Likewise, the coriander extract performed well by inhibiting the swarming motility of the bacterium. These results suggest the presence of inhibitors of the *las*, *rhl* and *pqs* inhibitors in both clove and coriander hexane extracts. However, the compounds found in the coriander and clove samples in the GCMS reveal a diversity in compound classes that could be used as inhibitors of QS. Typical compounds such as eugenol and linalool could not be detected in the GCMS when these compounds have been well recorded to be in abundance in both clove and coriander, respectively.

In summary, *Syzygium aromaticum* (clove) and *Coriandrum sativum* (coriander) are good samples in which various compounds can be found to further studies on bacterial infections, focusing on quorum sensing. A more focused investigation regarding the mode of action of these compounds is needed as this knowledge could help alleviate the rate at which multidrug-resistant bacteria emerge. Another decisive argument to encourage these investigations is about toxicity. Although coriander and clove form part of the diet to many people around the world, it is primordial to rule out or minimize any form of adverse effects before proceeding to clinical trials.

4.5. References

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CHAPTER 5 - CONCLUSION AND FUTURE RECOMMENDATIONS

5.1. General discussion and conclusion

Culinary herbs have been part of our diet for centuries, primarily for adding flavour to food and for medicinal purposes as well to treat various ailments. There is strong evidence that these herbs provide antioxidant properties, anti-inflammatory properties, anticarcinogenic properties, antibacterial properties and many more beneficial properties. Extensive research has made many advancements in finding bioactive compounds that could help eradicate bacterial infections. With antibiotic resistance on the rise, new strategies are needed. These strategies focus in targeting specific pathways that will lead to better treatment efficiency without creating a selection pressure. One important pathogen that is highly resistant to antibiotics is *Pseudomonas aeruginosa*. It is responsible for a large portion of nosocomial infections and affect patients with immunosuppressed systems, patients with cancers as well as patients affected by cystic fibrosis. *P. aeruginosa* is commonly used in research and is a pathogen that has been well studied. The knowledge of how this pathogen becomes virulent is well documented, which makes it easy to develop effective strategies to target specific pathways. This pathogen becomes virulent through quorum sensing that is regulated by four interconnected systems (*las*, *rhl*, *pqs* and *iqs*). The strategy of this study focused in selecting various culinary herbs to determine the presence of compounds that would interfere with one of these systems, consequently inhibiting the virulence of this pathogen.

Various extracts were made from these herbs and these extracts were tested for antibacterial activity through MICs against *P. aeruginosa*. All extracts displayed antibacterial activity with eleven extracts displaying noteworthy activity (activity at 1.00 mg/ml or less).

The extracts were also subjected to violacein inhibition activity using *Chromobacterium violaceum* 12472 (CV12472), a bacterium commonly used in quorum sensing studies. Only 2 extracts were able to inhibit the violacein production:

Coriandrum sativum (coriander) and *Syzygium aromaticum* (clove) hexane extracts. These two hexane extracts were able to reduce violacein by 24% (clove) and 69% (coriander).

These two extracts were further tested against *P. aeruginosa* virulence factors. Since virulence factors are linked to quorum sensing, a decrease in virulence factors activity would indicate the interference with quorum sensing system. Both clove and coriander inhibited biofilms formation by 70.3% and 80.1% respectively, at 1.00 mg/ml, highlighting the presence of compounds that interfere with the quorum sensing systems of this pathogen. Additionally, both extracts inhibited twitching motility by 43% (clove) and 36% (coriander) at 1.00 mg/ml. Furthermore, only the coriander extract was able to inhibit swarming motility by 35% at 1.00 mg/ml.

Moreover, elastase and pyocyanin inhibition assays were performed. Only the clove extract was able to decrease the elastase activity by 59% at 1.00 mg/ml whereas the coriander extract could not interfere with elastase. Also, both extracts did not have significant activity against pyocyanin activity. Despite this, there was evidence that these two extracts could be effective to discover new compounds that could explain the antivirulence properties against *Pseudomonas aeruginosa*.

The two hexane extracts of clove and coriander were subjected to gas chromatography. This resulted in the identification of 27 compounds from different classes. However, there were some important volatiles that are commonly found in these herbs that could not be obtained in this study. This was most likely due to the evaporation of these important volatiles, over the course of the study. Despite this event, the compounds identified possess some potential in interfering with quorum sensing, however speculation can only be made by performing molecular docking studies.

These observations provide evidence regarding the effectiveness of the extracts of the clove and coriander. With this knowledge, clove and coriander represent good candidates for further studies to understand all the specific mechanisms involved in their antiquorum sensing properties. Given that these two samples are often used in cuisine and flavouring, they could potentially be easy to access for more extensive research.

The objectives set in section 1.6 were achieved.

5.2. Future Work

From the study conducted, the following recommendations are made:

- To perform molecular docking and molecular dynamic simulations in order to speculate on possible mechanisms of action of compounds in the active extract
- Toxicity studies to perform to eliminate any risks or adverse effects of active compounds