

**ANTIPATHOGENIC PROPERTIES OF SELECTED SOUTH AFRICAN
MEDICINAL PLANTS TO COMBAT MULTIDRUG-RESISTANT BACTERIA**

By

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DECLARATION

I Itumeleng Tsebang Baloyi declare that this thesis entitled “Antipathogenic properties of selected South African medicinal plants to combat multidrug-resistant bacteria” which I hereby submit for the degree of Doctor of Philosophy in Microbiology at the University of Pretoria, is my work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature

Date:

DEDICATION

This thesis is dedicated to my nephews and nieces, hard work and dedication can get you where you need to be.

“If you can’t fly then run; if you can’t run then walk; if you can’t walk then crawl, but whatever you do, you have to keep moving forward”

Martin Luther King Jr.

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ABSTRACT

South African native plants have been predominantly explored for their antibacterial properties, overlooking their antipathogenic and antivirulence (also known as antiquorum sensing) potential. The bacterial quorum sensing (QS) system controls the virulence, biofilm formation and pathogenesis and collectively promotes diseases caused by multi-drug resistant (MDR) bacteria. Therefore, blocking the QS system may aid the management of various infectious diseases caused by human pathogens. Plant secondary metabolites can thwart bacterial colonization and virulence. Hence, this research was undertaken to explore the phytochemical, antivirulence or antipathogenic properties, molecular modeling and safety use of bioactive South African medicinal plants against selected MDR bacteria. Ten medicinal plants namely *Acokanthera oppositifolia* (Lam), *Aloe arborescens* (Mill), *Artemesia afra* (Jacq. ex Willd), *Lippia javanica* (Burm. f. Spreng), *Melianthus comosus* (Vahl), *Pelargonium sidoides* (DC), *Plectranthus ecklonii* (Benth), *Tetradenia riparia* ((Hochst.) Codd), *Trichilia emetica* (Vahl) and *Vachellia karroo* (Hayne. Banfi and Glasso) were selected based on their therapeutic use for various treatments such as skin infections, respiratory diseases and urinary tract infections (UTI). Plants were collected at Manie van der Schijff Botanical Garden, extracted using solvents with varying polarities then determined their minimum inhibitory concentrations (MIC) using the microdilution method against five MDR bacteria (*Streptococcus pyogenes*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Escherichia coli*). From the 50 plant extracts, *Melianthus comosus* (dichloromethane, aqueous and methanol), *Pelargonium sidoides* (aqueous) and *Vachellia karroo* (aqueous and methanol) extracts showed potent MIC values between 0.19 - 0.78 mg/mL against the five tested bacterial pathogens. A selected bio-monitor strain of *Chromobacterium violaceum* (ATCC 12472) with a violacein phenotype was used to assess plant extracts for their antiquorum sensing activities. The violacein production in *C. violaceum* was reduced up to 38.34% by *M. comosus*, *Plectranthus ecklonii* and *P. sidoides* extracts. The plant extracts that exhibited promising activities (antibacterial and antiquorum sensing), warranted further investigation for the identification of the compounds by using gas chromatography-mass spectrophotometry (GC-MS). Chemical

characterization of six active extracts of *M. comosus* (dichloromethane, aqueous and methanol), *P. sidoides* (aqueous) and *V. karroo* (aqueous and methanol) revealed compounds such as α -D-glucopyranoside, methyl, guanosine, neophytadiene, phytol, hexadecanoic acid and linalool, octadecanoic acid. An *in-silico* approach was then conducted to validate the antiquorum sensing properties of the compounds through the QS regulator protein (CviR') of *C. violaceum*. Sucrose, guanosine, α -D-glucopyranoside, methyl, phytol and 1,2,3-benzenetriol exhibited good docking scores between -7.59 and -5.57 kcal/mol for the CviR' protein of *C. violaceum*. The active plant extracts and phytochemicals were assessed on their effect to decrease the biofilm formation of selected MDR bacteria and their cytotoxic effect on the African monkey kidney Vero (epithelial). Treatment of five MDR bacterial pathogens with plant extracts and compounds for anti-adhesion and biofilm development showed up to 78.88% and 31.82% inhibitory effects, respectively. *In-vitro* cytotoxicity activities of plant extracts were shown to reduce the Vero cell's viability in a dose-dependent manner, showing a cytostatic effect, particularly at higher concentrations. Whilst both compounds of guanosine and phytol caused no cytotoxic effect on Vero cells. *Pseudomonas aeruginosa* was used as an MDR model pathogen to ascertain the molecular QS mechanism of the compounds. Two *P. aeruginosa* QS regulator proteins of LasR (2UV0) and PqsR (4JVD) were investigated through *in-silico* studies. Phytochemical compounds of sucrose, guanosine, α -D-glucopyranoside, methyl, phytol and 1,2,3-benzenetriol showed varying docking scores between 5.66 - 8.37 kcal/mol and 7.79 - 9.13 kcal/mol for 2UV0 and 4JVD proteins, respectively. Further, molecular dynamics simulation (MDS) findings showed phytol was the most stable compound that bound to the 2UV0 protein with an average root-mean-square deviation (RMSD) value of 1.47Å indicating a good solution whereas guanosine was the most stable for the 4JVD protein with an RMSD value of 2.10Å. The binding of all compounds resulted in a more flexible 4JVD conformation than the 2UV0. The *in-silico* findings were validated by targeting several *P. aeruginosa* virulence properties. *M. comosus* extracts and guanosine showed a significant reduction of pyocyanin production and inhibited swarming and swimming motility at a higher concentration. Overall, guanosine was the ideal potential QS compound that could be a candidate in search of antivirulent or antipathogenic drugs to combat bacterial infections caused by MDR bacteria.

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LIST OF ABBREVIATIONS

ADMET	Absorption, distribution, metabolism, excretion, and toxicity
AHLs	Acyl-homoserine lactones
<i>Agr</i>	Accessory gene regulator
Als	Autoinducers
AI-2	Autoinducer-2
AIPs	Autoinducing peptides
AMR	Antimicrobial resistance
AQS	Antiquorum sensing
BBB	Blood-brain barrier
BSTFA	N-O-bis(trimethylsilyl) trifluoroacetamide
C6-AHL	N-hexanoyl-L-acyl homoserine lactone
C8-AHL	N-octanoyl-L-Homoserine lactone
C10-HSL	N-decanoyl-homoserine lactone
CADD	Computer-aided drug design
cAMP	Cyclic adenosine monophosphate
c-di-GMP	Cyclic dimeric guanosine monophosphate
CC	Cytotoxicity concentration
CCK	Cell counting kit
CMEG	Centre for Microbial, Ecology and Genomics
CLSI	Clinical and laboratory standards institute
CV	Crystal violet
DNA	Deoxyribonucleic acid
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
EPS	Extracellular polymeric substances
ESKAPE	<i>Enterococcus faecium</i> , <i>Staphylococcus aureus</i> , <i>Klebsiella pneumoniae</i> , <i>Acinetobacter baumannii</i> , <i>Pseudomonas aeruginosa</i> , and <i>Enterobacter</i> spp
GC-MS	Gas chromatography-mass spectrophotometry

GI	Gastrointestinal
GNB	Gram-negative bacteria
GPB	Gram-positive bacteria
GPU	Graphic processing unit
HTS	High throughput screening
INT	P-iodonitrotetrazolium
LB	Luria Bertani
LDH	Lactate dehydrogenase enzyme
LSD	Least significant difference
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
MD	Molecular docking
MDS	Molecular dynamics simulation
MDR	Multi-drug resistant
MH	Mueller Hinton
MSTFA	N-methyl-N-(trimethylsilyl) trifluoroacetamide
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
NADH	Nicotinamide adenine dinucleotide hydrogen
OD	Optical density
OM	Outer membranes
PAINS	Pan assay interference compounds
PDB	Protein Data Bank
PBS	Phosphate-buffered saline
RMSD	Root-mean-square deviation
RMSF	Root-mean-square fluctuation
SAR	Structure-activity relationship
SAS	Statistical Analysis System
SEM	Scanning electron microscope
SI	Selectivity index
TIC	Total ion chromatogram

TPSA	Topological polar surface area
QS	Quorum-sensing
QSI	Quorum sensing inhibition
QSS	Quorum-sensing systems
VS	Virtual screening
WST	Water-soluble tetrazolium salt
WHO	World Health Organization
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

CHAPTER ONE

GENERAL INTRODUCTION & LITERATURE REVIEW

1.1. Introduction

Globally, microbial diseases caused by multidrug-resistant (MDR) bacteria have significantly impelled the high rates of mortality and morbidity, triggering a menace to global health (Borges et al., 2017). Among the MDR bacteria, the most opportunistic and frequent, associated with nosocomial infections are *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus pyogenes* while several other MDR bacteria predominantly cause community-acquired infections (van Duin & Paterson, 2017). Furthermore, several of these MDR pathogens form also part of ESKAPE pathogens including *Enterococcus faecium*, *S. aureus*, *K. pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa* and *Enterobacter* spp able to evade the bactericidal effect of antimicrobial agents (Mulani et al., 2019). The World Health Organization (WHO) estimated that by 2030, antimicrobial resistance (AMR) could compel the world into a financial burden, particularly the developing countries and by 2050 drug-resistant infections might cause 10 million deaths annually¹. This poses an immense medical crisis and challenge within the scientific community (Akbar et al., 2020).

The post-antibiotic era is imminent due to the escalating rates of antibiotic-resistant superbugs causing persistence in infections and aggravated due to their biofilm-forming ability (Borges et al., 2016; Mahavy et al., 2020). Biofilms are described as accumulates of microbial cells, that are encircled in an extracellular polymeric substances (EPS) (Ulrey et al., 2014). The occurrence of biofilm formation is influenced by living and non-living factors and various virulence genes in a cell density-dependent behaviour by quorum-sensing (QS), a regular communication system in bacteria (Kostylev et al., 2019). Bacterial cells within the biofilm are capable of eluding the host defense and can withstand antimicrobial therapies (Thi, Wibowo & Rehm, 2020). The bacteria form biofilms in the host, which makes the bacteria tolerant to antibiotic treatment and the action of the host defence system (Bjarnsholt et al., 2005). The development of biofilms is controlled by QS and has been revealed to regulate the tolerance of biofilms to antibiotic treatment (Høiby et al., 2011). Various strategies for non-antibiotic therapies need to be established for

¹ <https://www.who.int/news/item/29-04-2019-new-report-calls-for-urgent-action-to-avert-antimicrobial-resistance-crisis>

a post-antibiotic era (Wang, Hsieh, et al., 2020). The difficulties of AMR are intricate, except there are solutions (Mulat, Pandita & Khan, 2019). Of late the use of the biotechnological field to combat bacterial infections has been improved (Kalia et al., 2019); thus, inhibition of QS pathways for antivirulence agents is imperative (Borges et al., 2016).

Medicinal plants and their phytochemicals are the revolutions of novel therapeutic agents that potentially regulate bacterial QS mechanisms as compared to the existing antibiotics (van Duin & Paterson, 2017). This is because, various bioactive plants and phytochemicals have been documented to thwart virulent factors and the biofilm formation of bacteria (Singh & Bhatia, 2018; Chadha, Harjai & Chhibber, 2021). Hence, researchers are focused on discovering potential compounds that have antivirulence properties and understanding their relationship between QSI and antimicrobial action.

1.2. Rationale of the study

The efficacy of antibiotics is vital for the management of infections, however, the upsurge of MDR bacteria requires novel therapeutics (Schultz et al., 2020). Various research has endeavoured to apprehend the molecular mechanism triggering antibiotic ineffectiveness in planktonic bacteria (Lu et al., 2019; Guzzo et al., 2020). However, the indiscriminate use of antibiotics contributes to MDR due to the selective pressure applied for them to persist. As a result, it is imperative to explore the regulation of bacterial virulence factors, instead of killing their bacterial growth (Guzzo et al., 2020).

The rationale for opting to disrupt QS is based on its mode of action that focuses on impeding virulence factors and biofilms, without enforcing selective pressure on the microbe. This is because selective pressure is considered as a main source of microbial resistance (Singh & Bhatia, 2018). Quorum sensing inhibition (QSI, also known as antiquorum sensing or AQS) thus becomes the alternative strategy that promises to challenge MDR bacteria by regulating virulence factors and respective pathogenicity (Alvarez et al., 2014). Although, there are probabilities that bacteria may also evolve resistance towards the QS inhibitors (Kalia, Wood & Kumar, 2014). However, García-contreras, Maeda & Wood, (2016) mentioned that bacterial resistance can still arise from best-known antivirulence compounds such

as brominated furanones produced from seaweed *Delisea pulchra*. The management on bacterial resistance towards QSI compel researchers to be precautionous while developing drugs against QSS (García-contreras, Maeda & Wood, 2016).

In African countries, there is vast plant biodiversity, which presents complex plant-based compounds with a broad spectrum of biological activities (Kuete, 2010; Mahavy et al., 2020). This makes it a potential source of innovative antivirulence and/or antipathogenic (QSI) therapies. South African medicinal plants have been greatly considered for their antibacterial activities, yet many plant species are still overlooked for their QSI potential (Cock, Mavuso & Van Vuuren, 2021). Medicinal plants are presently receiving attention in the quest for plant-derived antivirulence or antipathogenic agents (Khan et al., 2018). Since time immemorial, medicinal plants have provided bioactive compounds as a source of antibacterial drugs, however, the assessment of these bioactives is laborious and costly (Abishad et al., 2021). Thus, the application of computer-aided drug design (CADD) techniques (virtual screening) decreases the cost and time of research in finding QSIs (Abdullahi & Adeniji, 2020). This further aids to comprehend the structure-activity relationship (SAR), mechanism of action, and protein-ligand interactions (Abishad et al., 2021).

1.3. Hypothesis

Plant-derived compounds can thwart bacterial colonization and pathogenicity of multi-drug resistant bacteria as a promising antivirulence therapy.

1.4. Aim

This study was undertaken to assess the antipathogenic potential, molecular modeling analysis and safety use of bioactive South African medicinal plants with their phytochemicals against selected MDR bacteria.

1.5. Research questions

This study intended to address the following questions:

- ❖ Which South African medicinal plants have potential use for the management of MDR bacteria, through modulating the bacterial QS?
- ❖ Which plant extracts and respective phytochemicals potentially reduce biofilm formation in selected MDR bacteria?
- ❖ *In silico* and *in vitro*, which phytochemical compounds meet the QSI criterion and hinder virulence factors, using *Pseudomonas aeruginosa*, an MDR model pathogen?
- ❖ Which of the bioactive extracts and phytochemicals are potentially safe for use, when evaluated for cytotoxic effect on normal epithelial with no malignant cells?

1.6. Objectives

To achieve the aim and answer the research questions raised above, the following specific objectives were met:

- ❖ Identification of suitable South African medicinal plants, *in-vitro* screening of respective plant extracts for antibacterial activities against selected multi-drug resistant bacteria, antiquorum sensing properties against *Chromobacterium violaceum* and chemical profiling of selected plant extracts.
- ❖ *In vitro* and *in situ* assessment of plant extracts and compounds for anti-biofilm activities against MDR bacteria
- ❖ *In vitro* proof-of-concept cytotoxicity evaluation of selected plant extracts and compounds on normal epithelial cells.
- ❖ *In silico* investigation of selected phytochemicals as QSI using *C. violaceum* (CviR) and *P. aeruginosa* (LasR and PqsR) as model pathogens.
- ❖ *In vitro* validation of the identified phytochemicals as potential antivirulent agents of *P. aeruginosa* (LasR and PqsR).

Prior to delving into the research approach and execution of this study, a comprehensive literature search was done to provide a succinct overview of MDR

bacteria, and QS mechanisms including their virulence, biofilm formation, and use of medicinal plants with their secondary metabolites as an alternative to combat bacterial infections.

1.7. Multi-drug resistant bacteria

The prevalence of resistance mechanism in MDR bacteria occurs and surpasses a definite limit of higher range antibacterials or adjuvants than an empiric therapy for community-acquired and nosocomial infections (van Duin & Paterson, 2017). The positivity of antibiotics has been dimmed as a result of progressed MDR mechanisms that pose a dire issue against frontline antibiotics (Alsheikh et al., 2020). As afore-mentioned, when antimicrobial drugs are used, they apply selective pressure that promotes bacterial viability and increases bacterial endurance (Kongkham, Prabakaran & Puttaswamy, 2020).

The MDR mechanisms used by bacteria that inactivate antibiotics include the integral expression of efflux pumps, biofilm formation, an adjustment in cell permeability modification of drug targets and production of a variety of enzymes that performs as a shield to antimicrobial agents, further promoting the tenacity of resistant bacteria (Alsheikh et al., 2020). Furthermore, mutations and portable genetic components such as integrative conjugative elements, insertion sequences, transposons and plasmids also have a key function in the development of resistance in numerous bacteria (Alsheikh et al., 2020). For example, Gram-negative bacteria (GNB) like *K. pneumoniae*, *P. aeruginosa* and *E. coli* commonly use intrinsic resistance mechanism as they have outer membranes (OM) that plays a significant role in minimal penetration of antibiotics and expression of efflux pumps (Ma et al., 2020).

Bacteria are known to develop rapidly due to their excessive modification rate, minute genomic size and gradual life span (Kongkham, Prabakaran & Puttaswamy, 2020). Hence the prominent focus should be on the drugs able to target the antagonistic effect on bacteria, that can reduce or hinder the increase in bacterial resistance (Mahavy et al., 2020). A diverse genus of bacteria uses the QS mechanism to coordinate their pathogenicity and virulence (Galloway et al., 2011).

1.8. Quorum sensing mechanisms

Quorum sensing defines a manner whereby bacteria detect their signalling molecules named autoinducers (AIs) (Dow, 2021). In a low cell density, the bacteria are unable to generate virulence factors and as a result, elude the host immune response (Lu et al., 2022). Once at a high threshold, the detection of AIs triggers the expression of genes essential for communal behaviours (Rutherford & Bassler, 2012). We now understand that a substantial part of the proteome ($\geq 20\%$) and bacterial genome (4–10%) are prompted by QS mechanisms (Prazdnova et al., 2022). Implying that pathogenic bacteria not solely regulate the activation of virulence factors but similarly for environment metabolic adaptation (Prazdnova et al., 2022).

Bacteria primarily use varied AIs for their QS processes which comprise acyl-homoserine lactones (AHLs), autoinducing peptides (AIPs) and autoinducer-2 (AI-2) with each performing a vital part in controlling bacterial pathogenicity (Jiang et al., 2019). The quorum-sensing systems (QSS) are composed of three steps: the signalling molecule synthesis, signal transduction and signal detection of molecules that each pathogen employs given their distinctive nature (Yüzbaşıoğlu et al., 2017). The key resemblances and variations in the systems used by GNB and GPB are briefly explained below.

1.8.1. Quorum sensing in Gram-negative bacteria

GNB utilise the LuxR-LuxI system with its linked signaling molecule AHL which is one of the greatly explored molecules and applied by numerous GNBs (Vega, Alvarez & McLean, 2015; Prazdnova et al., 2022). Figure 1.1 illustrates the prevalent Lux system which comprises a signal (AHL) generator LuxI homologue that generates the AHLs and LuxR homologue (response regulator) that binds with the AHLs to form AHL-receptor complexes, to trigger transcription of target QS-regulated genes (Asad & Opal, 2008; Husain et al., 2016). The LuxI/LuxR homologues are present in several pathogens linked to plant, animal and human infections, and are responsible for the modulation of motility, biofilm formation, DNA transfer, antibiotics production and virulence factors ((Yüzbaşıoğlu et al., 2017). Each bacterial pathogen applies its specific QSS like CviI/CviR in *C. violaceum* or

LasI/LasR, RhII/RhIR, PqsR and IQS in *P. aeruginosa* to generate their virulence factors (Rutherford, Bassler, 2012).

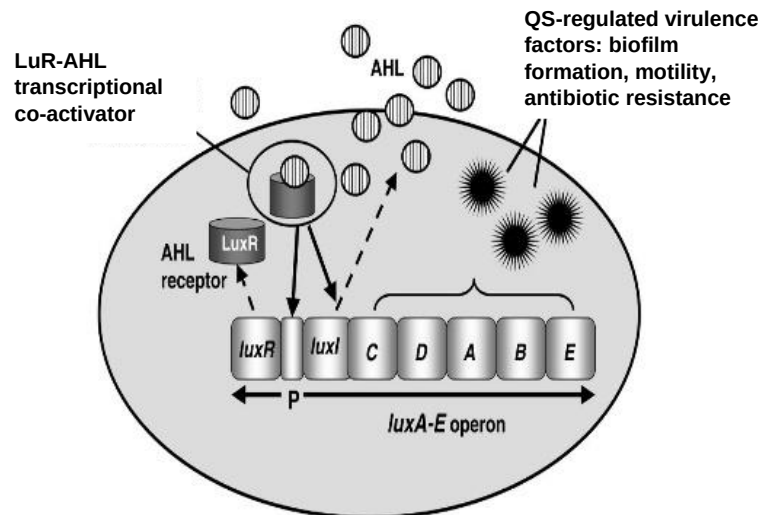


Figure 1.1: A typical LuxR-LuxI quorum sensing system in Gram-negative bacteria, LuxI protein activates the production of acyl-homoserine lactone (AHL) and then binds AHL molecules to the LuxR protein to activate gene expression of quorum sensing-related virulence factors (Asad & Opal, 2008).

Ahmed et al. (2021) mentioned that there are several bacterial species recognised to encode LuxR/LuxI homologues that control many genetic activities. However, the LuxR/LuxI QSS for *E. coli* and *K. pneumoniae* are indistinctly distinguished as they have a LuxR homologue called SdiA which demonstrates structural resemblance to the LuxR transcriptional receptor and it lacks the gene encoding of the LuxI homologue (Kim et al., 2014; Ahmed et al., 2021). Presently, a vast number of innate AHLs reveal similarities in structures, although individual bacterial species responds exactly to its particular distinctive AHL autoinducer (Galloway et al., 2011).

1.8.2. Quorum sensing in Gram-positive bacteria

GPB mainly uses two-component QSS comprising of a membrane-bounded histidine kinase receptor and a receptive controller. AIPs are predominantly carried externally out of the cell and identified by a sensor kinase protein within the membrane (Wang & Muir, 2016), then the triggered sensor kinase phosphorylates

the transcriptional factor and coheres to the promoter gene to control gene expression as illustrated in Figure 1.2 (Mulat, Pandita & Khan, 2019).

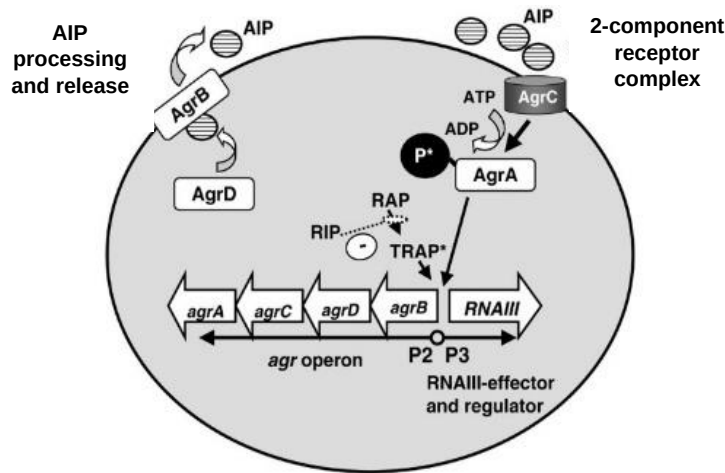


Figure 1.2: A typical autoinducing peptide (AIP) two-component quorum sensing system in Gram-positive bacteria. AIPs are divided, altered, and transferred via designated transporters. The AIPs (circles) are then sensed by two-component sensor-histidine kinases, and sensory data is conveyed through phosphorylation of cognate response regulators that control specific gene expression (Asad & Opal, 2008).

Other components of AIP-facilitated signalling systems involve the transcriptional regulators, efflux AIP transport systems, AIP synthases and activators which are similarly deemed as targets (Ivanova, Fernandes & Tzanov, 2013). Hindrance with AIP-facilitated QSS in GPB depends on the distinct targets. (Ivanova, Fernandes & Tzanov, 2013). For instance, in *S. aureus*, most of its virulence gene expression of enzymes, toxins and adhesins are activated by the accessory gene regulator (*agr*) system (Tan et al., 2018). *S. pyogenes* applies several QSS such as Sil, RopB (Rgg1), Rgg2/3 and ComR (Rgg4), with the Rgg2/3 as the commonly used QSS (Aggarwal et al., 2014).

1.8.3. Virulence factors and biofilm formation involved in pathogenic bacteria

Virulence is typically specified as the microorganism's capability to enter a host, reproduce and persist on the site for infection (Neville & Jia, 2019). MDR bacteria are linked with persistent infections across healthcare settings, frequently involved in biofilms and other virulence factors (Mishra et al., 2020). QS controls several

group behaviours such as swarming, biofilm formation, motility, antibiotic production bioluminescence, sporulation, toxins, siderophores and other virulence factors (Rutherford & Bassler, 2012). Various pathogens produce different virulence factors, for instance, *P. aeruginosa* (GNB) releases factors such as lectin, an exotoxin, elastase and pyocyanin (Jiang et al., 2019) whereas GPB, *Staphylococcus aureus* generates enterotoxin, protein A, fibronectin-binding protein, lipase and hemolysin that each performs a function in the pathogenicity of the bacteria (García-contreras, Maeda & Wood, 2013).

Bacteria grow on non-living surfaces or tissues and self-produce a matrix composed of nucleic acids, proteins, lipids, a variety of ions, water and exopolysaccharides (EPS) used to survive in an enclosed setting (Bramhachari, 2019). EPS performs a significant function with biofilm development thus enhancing resistance against conventional treatment (Srinivasan et al., 2016). Kumar, Mody & Jha, (2007); Rabin et al. (2015); Gopu & Shetty, (2016) recommends that the impediment of EPS production with treatments could potentially reduce and diminish structured biofilms of bacteria (Srinivasan et al., 2016). Considering that bacterial population can either exists as one or multiple genera which possibly have dissimilar phylogenetics (Schultz et al., 2020).

An example is patients suffering from cystic fibrosis, with their lungs infected with biofilm-forming *P. aeruginosa* and *Burkholderia cepacia* (Munir et al., 2020). Clinical available antibiotic therapies are practically not viable to eliminate biofilm infections (Wu et al., 2014). The *in vitro* and *in vivo* analyses showed that the minimum bactericidal concentration (MBC) and the minimum inhibitory concentration (MIC) for biofilms were a thousand times greater than the free-floating cells (Wu et al., 2014). Signifying diverse pathogens can cohabit and develop a collective of multi-species in the host (Munir et al., 2020). The cross-talk aspect between varying bacteria should also be greatly explored. Numerous secondary metabolites are known to have antivirulence activities at their sub-inhibitory concentrations as they can reduce the expression of virulence factors involved in causing infection (Díaz-Nuñez, García-Contreras & Castillo-Juárez, 2021). Serving as efficacious candidates for future medicines, as plant-based metabolites disrupt bacteria in various approaches (Alsheikh et al., 2020).

1.9. Medicinal plants as a potential source of antivirulence/antipathogenic agents

Medicinal plants have been a traceable source in finding antibacterial compounds and from the same plants, antivirulence compounds can be acquired (Díaz-Nuñez, García-Contreras & Castillo-Juárez, 2021). Mahavy et al. (2020) indicated that there are approximately 100 active compounds in the inventory of drugs from the African flora, whereas with Traditional Chinese Medicine (TCM) there are much greater than 2000 active compounds (Ehrman, Barlow & Hylands, 2007), suggesting an unparalleled possibility for the innovation of novel drugs (Mahavy et al., 2020). Africa is endowed with more than 60,000 taxa, which is equivalent to approximately 25% of the world's higher plant species, and a significant portion of these plants have therapeutic potential (Moyo, Aremu & van Staden). Several studies by Mulat, Pandita & Khan, (2019); Cosa et al. (2020); Baloyi et al. (2022); Mashamba et al. (2022) demonstrated that diverse plant parts utilised for the treatment of diseases within conventional medicinal practices can hinder the growth and virulence of numerous bacteria. Antiquorum sensing is one of the alternative strategies to examining antimicrobial activity however exploring the antipathogenic properties by quorum sensing has primarily been overlooked for South African medicinal plants (van Vuuren & Holl, 2017).

The focus is on identifying QSI compounds from frequently used medicinal plants to treat a variety of illnesses such as respiratory tract infections, gastrointestinal problems, skin problems, septic wounds and sores (Eloff, Angeh & McGaw, 2018). As presented in Figure 1.3, South African medicinal plants such as (a) *Acokanthera oppositifolia* (Lam), (b) *Aloe arborescens* (Mill), (c) *Artemesia afra* (Jacq. ex Willd), (d) *Lippia javanica* (Burm. f. Spreng), (e) *Melianthus comosus* (Vahl), (f) *Pelargonium sidoides* (DC), (g) *Plectranthus ecklonii* (Benth), (h) *Tetradenia riparia* ((Hochst.) Codd), (i) *Trichilia emetica* (Vahl) and (j) *Vachellia karroo* (Hayne. Banfi and Glasso) have long been used in traditional medicine and antipathogenic properties have not been specified. *L. javanica* (Maroyi, 2017a), *V. karroo* (Maroyi, 2017b) and *M. comosus* (Eloff, Angeh & McGaw, 2018; Maroyi, 2019) are some of the plants that have been extensively studied for their phytochemistry, ethnobotanical use, pharmacological effect, biological activities and commercial use, hence there is a need to also assess their antipathogenic

properties. Several classes of phytochemical compounds have also been identified from these plants such as terpenes, phenolic compounds and flavonoids (Kolodziej, 2007; Brendler & van Wyk, 2008; Nyila et al., 2010; Maroyi, 2017b; Eloff, Angeh & McGaw, 2018).

Plants have a vast number of structurally diverse compounds, individually having a different role in the defense of the plant (Alsheikh et al., 2020). Table 1.1. presents a synopsis of several QS inhibitors discovered from natural products such as cinnamaldehyde (Topa et al., 2020), quercetin from rich onion extracts (Quecan, Santos, Rivera, Hassimotto, Almeida & Pinto, 2019), hibiscus acid isolated from *Hibiscus sabdariffa* (Cortes-López et al., 2021), oilseeds (Pérez-López et al., 2018) and sedanenolide (Cosa et al., 2019) that have demonstrated to weaken bacterial pathogenicity and virulence are presented. When plants are exposed to the AHL they can stimulate varying physiological and molecular actions, important for influencing plant-microbe relations. These plants produce AHL mimickers that can hinder bacterial QS (Veliz-Vallejos, Kawasaki & Mathesius, 2020). Despite the that QS appears to be at the assembly point of bacterial virulence expression, QSS could be utilised differently by various bacterial species indicating that antivirulence agents will typically be strain-specific (Mahavy et al., 2020). Therefore understanding the mode of action before the development of QSI, especially with pathogenic bacteria is vital as QS is commonly dependent on the ligand-receptor network interaction (Munir et al., 2020).

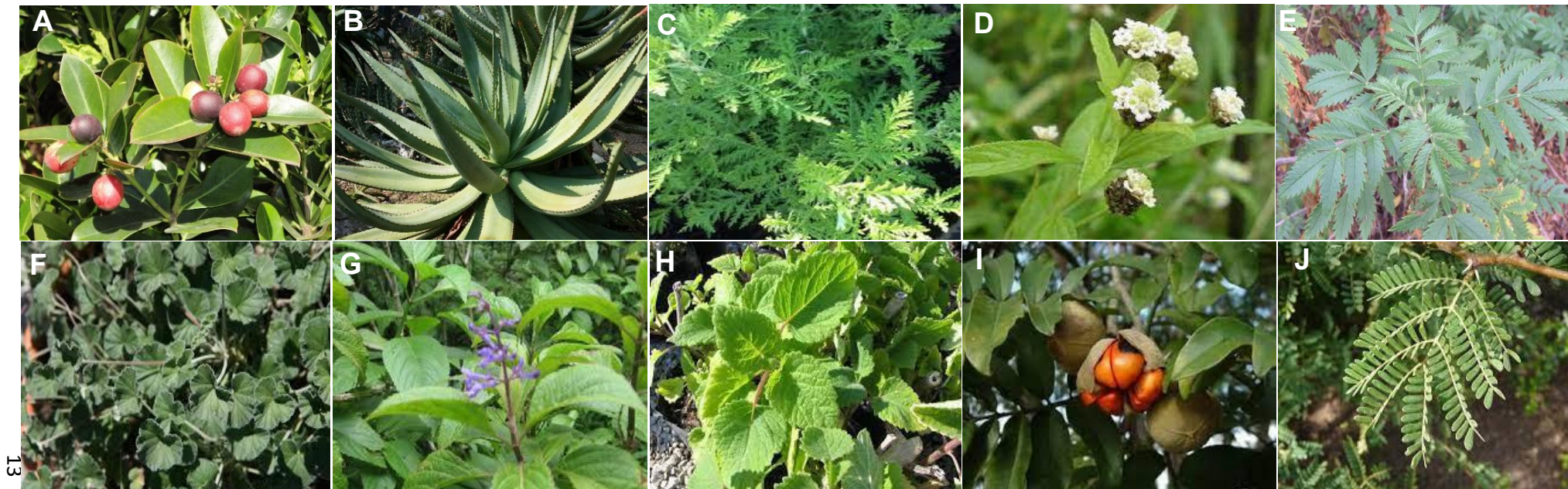


Figure 1.3: Medicinal plants used in traditional medicine in South Africa to treat various ailments. (a) *Acokanthera oppositifolia*², (b) *Aloe arborescens*³, (c) *Artemisia afra*⁴, (d) *Lippia javanica*⁵, (e) *Melianthus comosus*⁶, (f) *Pelargonium sidoides*⁷, (g) *Plectranthus ecklonii*⁸, (h) *Tetradenia riparia*⁹, (i) *Trichilia emetica*¹⁰ and (j) *Vachellia karroo*¹¹

² <https://www.randomharvest.co.za/en-us/South-African-Indigenous-Plants/Show-Plant/PlantId/117/Plant/Acokanthera-oppositifolia> [Accessed 29/08/2022]

³ <http://pza.sanbi.org/Aloe-arborescens> [Accessed 29/08/2022]

⁴ <http://pza.sanbi.org/Artemisia-afra> [Accessed 29/08/2022]

⁵ <http://pza.sanbi.org/Lippia-javanica> [Accessed 29/08/2022]

⁶ <http://pza.sanbi.org/Melianthus-comosus> [Accessed 29/08/2022]

⁷ <https://www.randomharvest.co.za/South-African-Indigenous-Plants/Show-Plant/PlantId/460?Plant=Pelargonium+sidoides> [Accessed 29/08/2022]

⁸ <http://pza.sanbi.org/Plectranthus-ecklonii> [Accessed 29/08/2022]

⁹ <http://pza.sanbi.org/Tetradenia-riparia> [Accessed 29/08/2022]

¹⁰ <http://pza.sanbi.org/Trichilia-emetica> [Accessed 29/08/2022]

¹¹ <http://pza.sanbi.org/Vachellia-karoo> [Accessed 29/08/2022]

Table 1.1: Summary of several quorum sensing inhibitors/antivirulence compounds identified from natural products

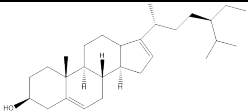
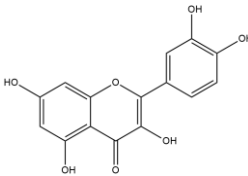
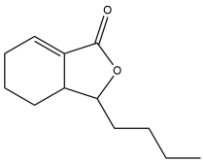
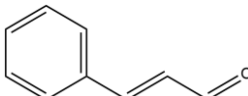
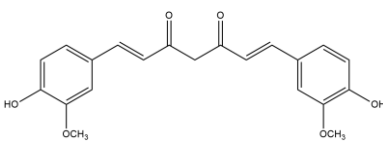
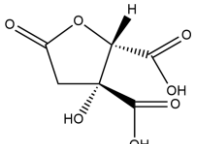
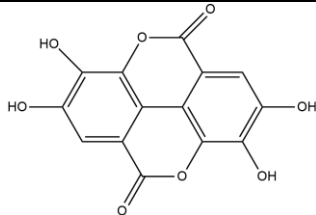
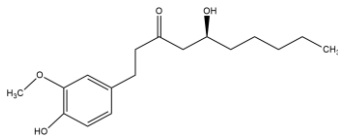
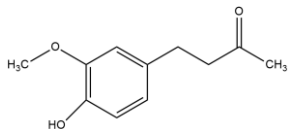
Plant species	Phytochemicals	Chemical structure	Antivirulence activities	References
<i>Allium cepa</i>	β -sitosterol		Reduction of pyocyanin and biofilm induction of <i>P. aeruginosa</i> .	(Al-Yousef & Sheikh, 2019)
	Quercetin		Inhibiting violacein production of <i>C. violaceum</i> and reduced biofilm biomass of <i>K. pneumoniae</i> and <i>P. aeruginosa</i> .	(Gopu, Meena & Shetty, 2015; Quecan, Santos, Rivera, Hassimotto, Almeida & Pinto, 2019)
<i>Apium graveolens</i>	Sedanenolide		Reduction of violacein production in <i>C. violaceum</i>	(Cosa et al., 2019)
<i>Cinnamomum zeylanicum</i>	Cinnamaldehyde		Reduces biofilm formation and virulence factor production of <i>S. pyogenes</i>	(Shafreen et al., 2014)
<i>Curcuma longa</i>	Curcumin		Reduces biofilm formation against <i>P. aeruginosa</i>	(Prateeksha et al., 2019)
<i>Hibiscus sabdariffa</i>	Hibiscus acid		Restricts swarming, alkaline protease, elastase and biofilm formation activities in <i>P. aeruginosa</i>	(Díaz-Nuñez, García-Contreras & Castillo-Juárez, 2021)

Table 1.1. Continued.

<i>Rubus ulmifolius</i>	Ellagic acid		Inhibits biofilm formation of <i>S. aureus</i>	(Quave et al., 2012)
<i>Zingiber officinale</i>	6-gingerol		Interferes with pyocyanin production, rhamnolipid, exoprotease, swarming, and biofilm formation of <i>P. aeruginosa</i>	(Kim et al., 2015)
	Zingerone		decreases motility, protease, biofilm formation, elastase, rhamnolipid, and pyocyanin, production phenotypes of <i>P. aeruginosa</i> PAO1	(Kumar, Chhibber, et al., 2015)

1.10. Overview of medicinal plants of interest

1.10.1. *Acokanthera oppositifolia*

Acokanthera oppositifolia (Lam.), Codd., an Apocynaceae, commonly known as inhlungunyembe (Figure 1.3.A). *A. oppositifolia* is an evergreen shrub with pinkish-white flowers and red berries which become dark purple when ripe (Adedapo et al., 2008). The leaves of this plant are used as an infusion for convulsions, septicaemia, and abdominal pains, and it is also used in form of snuff for treating headaches (Hutchings et al., 1996) (Table 1.2). This plant species is usually distributed in the South African regions of Gauteng, KwaZulu Natal, Mpumalanga, Western Cape, North West, Eastern Cape and Limpopo provinces. *A. oppositifolia* possesses antimicrobial, antioxidant and cytotoxicity activities (Mabona et al., 2013). Several phytochemical compounds like acovenoside A, acofrioside L and acolongifloroside H have been identified from this plant (Saeed et al., 2016).

1.10.2. *Aloe arborescens*

Aloe arborescens (Mill.), a Xanthorrhoeaceae commonly known as krantz aloe or umhlabana (Figure 1.3.B). Krantz Aloe is a tall single-stemmed with curved thorny leaves arranged in a rosette and has orange flowers (Sserunkuma et al., 2017). *A. arborescens* is indigenous to South Africa and grows in the Eastern and Western Cape, KwaZulu Natal, Gauteng and Limpopo provinces. This plant is medicinally used for colds, fever, asthma, stomach ache, burn wounds, abrasions, bruises and gastrointestinal ailments (Hutchings et al., 1996) (Table 1.2). *A. arborescens* has been reported to have antimicrobial, antifungal, antiinflammatory and cytotoxicity activities and contains phytochemicals such as phenols, anthraquinones and aloin (Ghuman et al., 2016; Sserunkuma et al., 2017).

1.10.3. *Artemisia afra*

Artemisia afra (Jacq. ex Willd.), an Asteraceae is a perennial woody shrub that grows in profuse and faintly cluttered clumps with soft leaves that are finely distributed more like a fern (Figure 1.3.C). *A. afra* is a common species, known as

mhlonyane and is widely distributed in all provinces of South Africa except in Mpumalanga (Liu, Van der Kooy & Verpoorte, 2009). Several ailments including respiratory tract infections including colds, pneumonia, coughs and gastrointestinal complaints are treated by this plant (Hutchings et al., 1996) (Table 1.2). *A. afra* has been well-studied with antimicrobial, antioxidant, and anti-inflammatory properties and contains various phytochemical compounds such as terpenes, coumarins and flavonoids (Liu, Van der Kooy & Verpoorte, 2009).

1.10.4. *Lippia javanica*

Lippia javanica (Burm. f. Spreng.), a Verbenaceae with vertical woody perennial herb or shrub with potent aromatic leaves when crushed emits a lemon-like scent (Figure 1.3.D). *L. javanica* is generally known as lemon bush and is used to treat gastrointestinal complaints, skin infections, rashes and respiratory infections like influenza, coughs, bronchitis and colds (Hutchings et al., 1996) (Table 1.2) and grows in all parts of South Africa except for the Western and Northern Cape. *L. javanica* contains various classes of compounds, namely iridoids, flavonoids, alkaloids, triterpenes and amino acids (Maroyi, 2017a). The plant species has been documented to have antibacterial, antifungal, antimalarial, antioxidant and cytotoxicity properties (Maroyi, 2017a).

1.10.5. *Melianthus comosus*

Melianthus comosus (Vahl), a Melianthaceae with perennial, evergreen and multi-stemmed shrub (Figure 1.3.E), commonly known as the honey flower. *M. comosus* is therapeutically used for skin problems, sores, snake envenomation, septic wounds, respiratory problems, backache, rheumatism and gastrointestinal problems (Hutchings et al., 1996) (Table 1.2). *M. comosus* is widespread across seven provinces of South Africa, namely: Northern Cape, Eastern Cape, Mpumalanga, Gauteng, Western Cape, Free State and North-West. This plant has been reported to have anti-inflammatory, antifungal, antioxidant, antibacterial, and cytotoxicity, also consist of triterpenoids, flavonoids, phytosterols, and glycosides (Almeida et al., 2017).

1.10.6. *Pelargonium sidoides*

Pelargonium sidoides (DC.), Geraniaceae commonly known as ikubalo is a rosette-like plant with crowded leaves that are heart-shaped and slightly aromatic (Figure 1.3.F). This plant is used for treating several ailments including bronchitis, cough, diarrhoea, fever and sore throats (Kumar et al., 2015). *P. sidoides* occurs in South African provinces of Mpumalanga, Free State, Eastern Cape, North West and KwaZulu-Natal and is traditionally used to treat rheumatic fever, diarrhoea, cough, bronchitis and tuberculosis (Hutchings et al., 1996) (Table 1.2). There are several phytochemical compounds present in *P. sidoides* including phenolics, coumarins, and flavonoids that have been shown to demonstrate antimicrobial, cytotoxic and antioxidant properties (Aboobaker et al., 2019).

1.10.7. *Plectranthus ecklonii*

Plectranthus ecklonii (Benth.), a Lamiaceae usually known as tall spur flower and is a delicate, upright, rapidly growing shrub with attractive spikes of pink, blue or white flowers (Figure 1.3.G). This plant is traditionally used for treating stomach aches, nausea, vomiting, meningitis, respiratory problems, chest complaints and coughs; grows in South African regions of Eastern Cape, KwaZulu-Natal and Mpumalanga (Nyila et al., 2010) (Table 1.2). *P. ecklonii* contains compounds like phenolics, terpenes and have anti-inflammatory, antimicrobial and antifungal properties (Nyila et al., 2012).

1.10.8. *Tetradenia riparia*

Tetradenia riparia (Hochst. Codd), a Lamiaceae known as the ginger bush, is an aromatic shrub with succulent, uneven branch pattern and bright green leaves (Figure 1.3.H). *T. riparia* occurs in Limpopo, KwaZulu-Natal, Mpumalanga and Gauteng in South Africa, this plant is normally used for treating ailments of influenza, skin conditions, mouth ulcers, and stomach complaints (Hutchings et al., 1996). This plant comprises terpenes and ketones, considered to have cytotoxic, antifungal and antimicrobial activities (Coopoosamy R & Naidoo K, 2011).

1.10.9. *Trichilia emetica*

Trichilia emetica (Vahl), a Meliaceae generally identified as Natal Mahogany an evergreen tree with dark glossy leaves and a wide-spreading crown (Figure 1.3.I). *T. emetica* is native to the South African provinces of Limpopo, KwaZulu-Natal and Mpumalanga and is used for the treatment of various ailments such as wounds, headaches, cough chest pain skin diseases and urinary tract infections (Hutchings et al., 1996) (Table 1.2). This plant is a rich source of triterpenoids and has been reported to have biological properties including anti-inflammatory, cytotoxic, antibacterial and antioxidant activities (Saeed et al., 2016).

1.10.10. *Vachellia karroo*

Vachellia karroo ((Hayne) Banfi and Glasso), a Fabaceae has a round crown, branched equally low down on the trunk with thin green leaves and yellow pompons flowers that usually show in the early summer (Figure 1.3.J). This species in South Africa is habitat in regions of Mpumalanga, Eastern and Western Cape provinces and is used to treat ailments like flu, colds, dysentery and wounds (Hutchings et al., 1996) (Table 1.2). *V. karroo* contains varying classes of phytochemicals including phenols, phytosterols, flavonoids and terpenes and this plant has been shown to exhibit antimalarial, antioxidant, antibacterial, antilisterial, antimycobacterial, anthelmintic and antifungal properties (Maroyi, 2017b).

Table 1.2: South African medicinal plants associated with treating nosocomial infections

Plant species	Common name	Family name	Medicinal use	Voucher specimen	References
<i>Acokanthera oppositifolia</i> (Lam.) Codd	Inhlungunyembe (Z)	Apocynaceae	Abnormal menstruation, septicemia, toothache	PRU 125454	Hutchings et al., 1996
<i>Aloe arborescens</i> (Mill)	Umhlabana (Z)	Xanthorrhoeaceae	Colds, fever, asthma, stomach ache, burn wounds, abrasions, bruises and gastrointestinal ailments	PRU 125455	(Chen, van Wyk, Vermaak and Viljoen, 2012)
<i>Artemesia afra</i> (Jacq. ex Willd)	Mhlonyane (Z)	Asteraceae	Coughs, colds, fever, respiratory tract infections, influenza, pneumonia, and gastritis	PRU 125456	Hutchings et al., 1996
<i>Lippia javanica</i> (Burm.f. Spreng.)	Lemon bush (E)	Verbenaceae	Fever, coughs, colds, bronchitis, stomach problems, influenza, and measles	PRU 125882	Hutchings et al., 1996
<i>Melianthus comosus</i> (Vahl)	Honey flower (E)	Melanthaceae	Septic sores and wounds, snakebite, backache, rheumatism, respiratory problems, skin problems and gastrointestinal problems	PRU 125457	Hutchings et al., 1996
<i>Pelargonium sidoides</i> (DC.)	Ikubalo (Xh)	Geraniaceae	Bronchitis, diarrhoea, cough, fever and other respiratory-related ailments	PRU 125883	Hutchings et al., 1996
<i>Plectranthus ecklonii</i> (Benth)	Tall spurflower (E)	Lamiaceae	Stomach aches, nausea, vomiting, meningitis, respiratory problems, chest complaints and coughs	PRU 125458	(Nyila et al., 2010)
<i>Tetradenia riparia</i> ((Hochst.) Codd)	Ibosa (Z)	Lamiaceae	Colds and flu, bronchitis, stomach upsets, flatulence, mouth ulcers, diarrhoea, and fevers are used externally, as an inhalation, for headaches.	PRU 125459	Hutchings et al., 1996
<i>Trichilia emetica</i> (Vahl)	Umathunzi (Z)	Meliaceae	Skin diseases, urinary tract infection, wounds, headache, cough and chest pain	PRU 125460	Hutchings et al., 1996
<i>Vachellia karroo</i> (Hayne. Banfi and Glasso)	Umunga (Z)	Fabaceae	alimentary canal, rheumatism, convulsions, gonorrhoea, syphilis, and diarrhoea	PRU 125884	Hutchings et al., 1996

Z: Zulu; Xh: Xhosa; E: Englis

1.11. Identifying antipathogenic agents

1.11.1. *In vitro* and/or *in vivo* screening of medicinal plants for antipathogenic properties

The *in-vitro* screening of medicinal plants with their phytochemicals has been widely investigated for their biological activities (Lu et al., 2019). The simplest methods to investigate suitable QSI include tests with biomonitor strains such as *Chromobacterium violaceum* with a violacein pigment and *Vibrio harveyi* producing bioluminescence (Lu et al., 2022). These QS phenotypic strains are used for preliminary screening to assess the antiquorum sensing activities of plant extracts and phytochemicals (Munir et al., 2020). Further *in-vitro* evaluation on QS-regulated phenotypes including biofilm formation inhibition and the antivirulence assay may considerably reduce the number of potential candidates (Lu et al., 2022). The various *in-vitro* assays applied provides an overview of the biological properties the plant extracts possess which give a direction towards a proper selection of bioactive extracts for further investigation of their phytochemical compounds. Although, the *in vitro* results should not be regarded as an end-point conclusion for clinical trials, rather it be a starting phase step and should be confirmed through *in vivo* studies (Seleem et al., 2020). QSI is also required to be further evaluated for its potential use on the human host (Bjarnsholt et al., 2010). Smyth et al. (2010) had a first clinical trial study on patients suffering from cystic fibrosis the treated with garlic (QSI) and indicated that garlic could possibly be a promising biomarker of *P. aeruginosa* for cystic fibrosis patients. *In vitro* and *in vivo* studies might not appear to sufficiently reveal that tested QSI particularly interrupt QS signaling ways thus the mechanisms and modes of action related to the inhibitory effects should be investigated (Lu et al., 2022).

1.11.2. Computer-based techniques

Virtual screening (VS) is an essential technique that applies computer-based procedures to visualise novel promising compounds. This focuses on minimising the massive computer-generated natural molecules lists (Maruca et al., 2019), thus ranking compounds accordingly to their chemical structures for the biological activity

(Maruca et al., 2019). Previously, drug innovation was a time-consuming and costly procedure due to a large number of compounds synthesized, to evaluate them for a specific activity (Suay-Garcia et al., 2020). Computer-based techniques offer a better resolution, permitting scientists to discover molecules that could be used as potential drugs, that are only quality for further relevant assays (Suay-Garcia et al., 2020).

Recently, 83.8% of the general protein structures in the Protein Data Bank (PDB) were generated through X-ray crystallography, providing an initial step to drug lead design (Neville & Jia, 2019). Innovations of computational tools and screening procedures for the improving number of confirmed virulence protein targets have made the logical layout, to delve into pursuing antivirulence agents (Neville & Jia, 2019). The pattern of *in silico* methods with improved purification, structural analysis and modernising the assessment procedure has lessened the timeline required for the screening of natural drugs for the management of diseases (Maruca et al., 2019).

Molecular docking (MD) in particular, is one of the tools used to comprehend and predict binding affinities for the identification of compounds that have both similar binding mechanisms and structures to the target protein (Morris & Lim-Wilby, 2006). MD analyses can be achieved by applying signaling molecules (native ligands) of a specific target protein within a bacteria and can be used to identify competitive ligands that bind to the receptor protein to understand their inhibitory effect (Aliyu et al., 2016). Docking analysis is further validated by using the molecular dynamics simulations (MDS) tool, which indicates the protein-ligand binding traits, stability and flexibility (Hein, Zilian & Sottriffer, 2010). By assessing important parts of active molecules using computer-based models, researchers can attain insightful knowledge of the molecular basis of antivirulence activities (Suay-Garcia et al., 2020). Researchers such as Shafreen et al. (2014); Aliyu et al. (2016); Singh & Bhatia, (2018); Quecan et al. (2019) have already applied MD studies to ascertain potential quorum sensing compounds and understand their structure-activity relationship. Abinaya & Gayathri, (2019); Pereira et al. (2019); Ahmed et al. (2021) have also shown the effectiveness of using molecular docking in exploring plant-derived compounds that further required to be explored through *in vivo* and *in vitro* studies. These *in silico* methods ought to be confirmed by *in vitro* and *in vivo*

studies, nevertheless, computational methods have gained interest in drug design production, and it is significant in drug repurposing (Mellini et al., 2019).

1.12. Concluding Remarks

Since the ever-evolving MDR bacteria and post-antibiotic era rapidly advanced the quest for novel therapeutics and more interest is focused on medicinal plants and their phytochemicals as an alternative and adjuvants for the failing antibiotics (Pan et al., 2013). Globally, evidence of the vital influence of natural products, especially plant materials in drug discovery, cannot be overemphasized (Gurib-Fakim, 2006). There is an improved and increasing research interest in investigating options such as natural products to focus on the dilemma and global emergence of MDR bacteria (van Vuuren & Muhlari, 2017). Thus far, bacterial QS mechanisms and regulation of virulence factors for novel antivirulent compounds is greatly explored (Asfour, 2018). The application of several techniques including computational methods generates insightful knowledge-based tactic which assists with the selection of natural products that have a high possibility for biological activity (Atanasov et al., 2015). Thus *in-silico* technique can be used as a starting point for the promising compounds for the *in-vitro* studies (Atanasov et al., 2015). Rationally designed antivirulence compounds can develop a vital defense mechanism against MDR bacteria. For those reasons, impeding bacterial QSS are a ground-breaking way for future work in increasing the establishment of QS compounds to thwart MDR infections.

The succeeding chapters will show the findings of these medicinal plants on their antibacterial and antiquorum sensing activities on selected MDR bacteria, identification of phytochemical compounds in bioactive extracts and their quorum sensing mechanisms and attenuation of virulence factors on selected MDR bacteria.

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CHAPTER TWO

***IN VITRO* ANTIBACTERIAL VALIDATION, QUORUM SENSING INHIBITION AND CHEMICAL PROFILING OF SELECTED SOUTH AFRICAN MEDICINAL PLANTS AGAINST MULTI-DRUG RESISTANT PATHOGENS**

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2.1. INTRODUCTION

Multidrug-resistant (MDR) bacteria gradually progress in the health care and public system, causing a threat globally (Karunanidhi et al., 2018). The predominant infections such as bacteraemia, intra-abdominal, urinary tract, pneumonia, skin and soft tissue are caused by pathogens such as *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes* and *Staphylococcus aureus* are related to multidrug resistance (Bhatia et al., 2021). Among bacterial pathogens, MDR is normally produced by the accrual of every gene coding evolving resistance to one drug, on R plasmids (Nikaido, 2009). Aforementioned, the escalated rate of MDR bacteria is caused by bacteria's ability to use efflux pump, expelling various, structurally dissimilar compounds from the cell, causing resistance to a vast selection of antibiotics (Tang, Apisarnthanarak & Hsu, 2014). Pathogenic bacteria exhibit resistance to numerous antimicrobials, thus finding innovative antipathogenic drugs or plant-based antimicrobials other than those isolated from bacteria continues (Munita & Arias, 2016). Moreover, the conventional approach of antibiotic exploration has decreased, directing us to a world with futile antibiotics (Mulani et al., 2019).

The emergence of microbial resistance to an immense types of antibiotics demands the focus on alternative target sites for the pathogen such as QS regulatory systems (Zhao, Yu & Ding, 2020). So far, the regulation of microbial diseases through interfering with QS mechanism could be essential by way of (i) competitively binding signal protein mimics to the receptor protein (ii) reducing the production of signal proteins, if not by (iii) destroying signal proteins (autoinducers) (Moradi, Hadi & Bazargani, 2020). There are alternatives that medicinal plants may possess or apply other processes like the antiquorum sensing (AQS) approach to inhibit infective bacteria (Bouyahya et al., 2017).

For numerous years, plant-based medicines are immensely made from extracts that comprise of a variety of phytochemicals (Konappa et al., 2020). These natural products essentially exert antibacterial, antiviral, and antifungal effects, responsible for the medicinal properties they revealed against pathogenic bacteria (Khan et al., 2018). This has stimulated the advancement of new drugs from naturally derived compounds or secondary metabolites, as they turn out to be the source for improving medication or treating infections (Konappa et al., 2020). The

quest to find novel plant-based antipathogenic agents is also due to their phytochemistry comprising of potential groups of compounds like tannins, benzoic acid derivatives, flavonoids, terpenoids and coumarins.

Chromobacterium violaceum is considered and accepted as the best suitable organism to screen plant extracts for their quorum sensing activities (Cosa et al., 2020). This is because *C. violaceum* produces a purple pigment recognised as violacein. The violacein production is regulated by the pathogen's QSS, hence it serves as a useful tool to understand the mechanism of action of numerous conventional therapeutic products (Pattnaik et al., 2018).

Here, following the validation of the antibacterial activities, we hypothesised that the 10 South African medicinal plants may have bioactives with the possibility to disturb QS signalling by any of the proposed approaches. These plants were chosen based on their known medicinal usage for the treatment of septicaemia, urinary tract infections, respiratory diseases, wounds and skin infections including their reported antibacterial, anti-inflammatory, antifungal and antidiabetic activities. Although South African traditional plants are prevalently recorded for antibacterial activities (Maroyi, 2019), their associated antipathogenic properties are limited. Furthermore, prior to this study, these medicinal plants of interest were not documented for anti-pathogenic or antivirulence properties.

In this chapter, 10 South African medicinal plants were validated for their antibacterial activities against MDR pathogens, assessed for their AQS potential, *in vitro* and performed chemical characterization analysis of bioactive extracts, elicited from their potent bioactivity against the tested pathogens.

2.2. MATERIALS AND METHODS

2.2.1. Plant collection and identification

The leaves of 10 medicinal plants were harvested at the Manie van der Schijff Botanical Garden (25° 45' 15.84" S, 28° 13' 43.2084" E) at the University of Pretoria, South Africa. The plants were positively identified by Ms Magda Nel from the Department of Plant and Soil Sciences, University of Pretoria. The voucher specimens were deposited at the H.G.W.J. Schweickerdt herbarium of the University of Pretoria, South Africa.

2.2.2. Plant preparation and extraction

After plant material collection, the leaves were washed and left to air-dry at room temperature for 3-7 days. Then, the dried-up leaves were ground into a fine powder using an IKA MF 10.1 cutting grinder (Cole-Parmer scientific experts, Chicago, United States of America (USA)). The plants were extracted using maceration (organic solvents) and decoction (aqueous) technique, following a similar approach according to Baloyi et al. (2019), with minor modifications. Twenty-five (25) grams of each fine powder per sample was added to 250 mL of the following solvents with varying polarities; methanol, acetone, ethyl acetate and dichloromethane and extracted for 48 h with agitation at 150 rpm using a 261-orbital shaker (Labotec, Johannesburg, South Africa). After 48 h, the extracts were filtered through a Whatman no. 1 filter paper (11 µm). The filtrates were dried using a Buchi rotary evaporator- interface I-100 (Labotec, Johannesburg, South Africa) at 50 °C. The extracts were transferred into 10 mL glass vials, further dried in a fume hood cabinet and stored at 4 °C until required for further analysis.

In parallel for aqueous extracts, a 25 g of powdered plant material was added to sterile distilled water (250 mL) and boiled for 15 min afterwards left to cool down before filtering. The filtrates were transferred to glass bottles and lyophilised using a freeze-drier (SP Scientific, New York, USA). All the dried extracts were weighed using a weighing balance (Kern 770, Microsep, Johannesburg, South Africa), re-dissolved in 1% aqueous dimethyl sulfoxide (DMSO) by sonication in a 701 ultrasonic bath (Labotec, Johannesburg, South Africa) and transferred to 10 mL vials. The stock solutions (100 mg/mL) for the 10 plant extracts were later diluted to the required concentrations (25 mg/mL and 1 mg/mL) for the biological assays. The percentage yields for each extract were calculated using equation 1.

$$\text{Percentage yield (\%)} = \frac{\text{dry crude extract}}{\text{dry initial material before extraction}} \times 100 \quad (1)$$

2.2.3. Bacterial strains and growth conditions

The bacterial strains: *Escherichia coli* ATCC 10536, *Klebsiella pneumoniae* ATCC 33495, *Pseudomonas aeruginosa* ATCC 9721, *Staphylococcus aureus* ATCC 25923 and *Streptococcus pyogenes* ATCC 19615 were purchased from Sigma-Aldrich (Johannesburg, South Africa). A bio-monitor strain *Chromobacterium violaceum* ATCC 12472 that generates QS-regulated purple pigment was used for the qualitative and quantitative determination of AQS which was kindly provided by the Centre for Microbial, Ecology and Genomics (CMEG), University of Pretoria, South Africa. Ethics approval for the use of these microorganisms was obtained from the University of Pretoria, Faculty of Natural and Agricultural Sciences Ethics Committee (reference number: NAS158/2021) see Appendix 1.

These pathogens were grown in their respective media and under incubation conditions for batch-batch reproducibility as described by the Clinical and Laboratory Standards Institute, (2020). The active bacterial cultures of *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus* and *S. pyogenes* were prepared in Mueller Hinton (MH) medium and incubated at 37 °C whereas *C. violaceum* was prepared in Luria Bertani (LB) medium and incubated at 30 °C.

For the maintenance of the bacterial strains, glycerol stock cultures of each organism were prepared and kept at -80 °C freezer until required. Before each assay, the bacterium was grown for 24 h at 30 °C and 37 °C on a respective agar plate. A single or two colonies were transferred to sterile distilled water to obtain an absorbance (OD_{600nm}) of 0.1. This adjustment of cell suspension was performed to achieve a 0.5 Mc Farland standard equivalent.

2.2.4. Antibacterial activity using a microdilution assay

The minimum inhibitory concentration (MIC) of the 10 extracts against *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus* and *S. pyogenes* were determined, using the microdilution method as previously described by Eloff, (1998), with minor modifications. Succinctly, 100 µL of Mueller Hinton broth (MHB) transferred into every well and 100 µL of each plant extract (in triplicate) was transferred into wells in Row A of the micro-titer plate together with the negative (1% dimethyl sulfoxide) and positive controls (ciprofloxacin) at starting concentration of 0.01 mg/mL. Also, a

blank (sterile MH broth) and standardised bacterium (control) were prepared by transferring 200 μ L to the wells, respectively. Two-fold serial dilutions were performed, resulting in decreasing concentrations over the range of 6.25 - 0.048 mg/mL.

Thereafter, 100 μ L of the standardised bacterium was added to each well. After 24 h incubation at 37 °C, 40 μ L of P-iodonitrotetrazolium (INT, 0.2 mg/mL) was added and incubated for a further 30 min to 1 h until the colour of the solution becomes pink. Bacterial growth inhibition (clear wells, no colour change) was evaluated visually (Figure 2.1) and recorded. The MIC was recorded as the lowest concentration of the extract that inhibited bacterial growth.

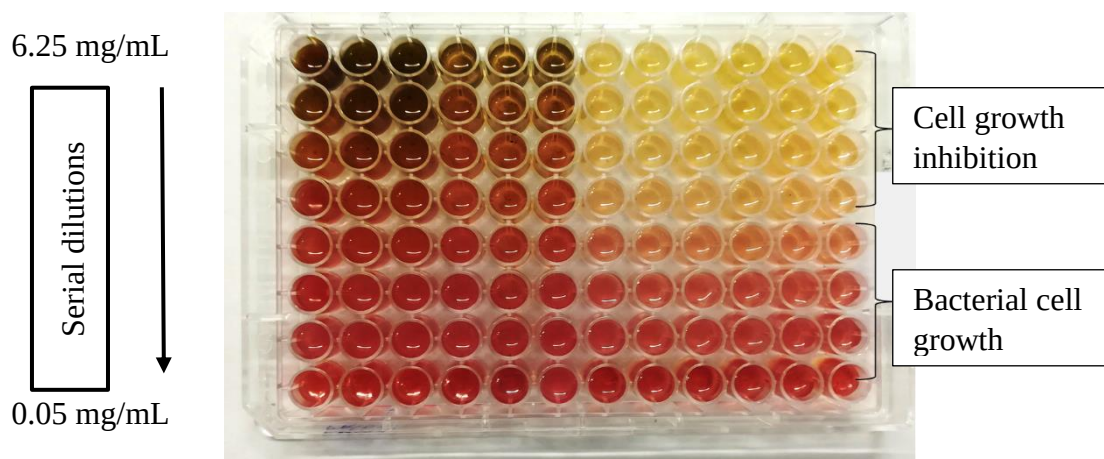


Figure 2.1: Interpretation of minimum inhibitory concentration (MIC) broth dilution assay. All wells with a pink-red solution indicate cell growth while clear wells with no colour change indicate a concentration of the cell growth inhibition. The lowest concentration of the extract that inhibited bacterial growth was the MIC value.

2.2.5. Evaluation of plant extracts for antiquorum sensing (AQS) potential

C. violaceum is well-accepted as a biomonitor strain for antiquorum sensing or antipathogenic agents screening due to its production of violacein being regulated by the organism's QS system. Furthermore, the violacein is visually observed in the bacterium's colonies, and hence its production can be tempered in various, consequently relatively altering its QS system. Furthermore, the violacein is visually observed in the bacterium's colonies, and hence its production can be tempered in

various, consequently relatively altering its QS system. Comparatively, of the two methods (qualitative and quantitative violacein assays) used to evaluate the QS inhibitory effects of the extracts, the modified microdilution assay is more appropriate in contrast with the disc diffusion assay. This may be attributed to the secondary metabolites in the plant extracts (Yüzbaşıoğlu et al., 2017).

2.2.5.1. Qualitative antiquorum sensing assay

The detection of AQS activity of the extracts was carried out using the disc diffusion assay, following the method described by Chenia, (2013), with minor modifications. Briefly, the bacterium was grown for 24 h at 30 °C on an LB agar plate and the bacterial suspension was adjusted to an OD_{600nm} of 0.1. The standardised culture was swabbed uniformly on the agar plate surface. The sterile discs (6 mm diameter) were impregnated with 10 µL of extracts with varying sub-MIC concentrations (6.25, 3.12, 1.56 and 0.78 mg/mL), with positive control of cinnamaldehyde (0.50, 0.25, 0.12 and 0.06 mg/mL) and incubated for 24 h at 30 °C. The plates were analysed for violacein production, whereby the loss of purple violacein pigment (opaque zone) surrounding the disc indicated AQS while clear and transparent zones indicated bactericidal activity around the discs. The zones displaying antimicrobial growth inhibition were measured as diameter 1 (d1) in mm, while the zones displaying both antimicrobial growth and pigment inhibition by plant extracts, were measured as diameter 2 (d2). The pigment inhibition was determined by subtracting the antimicrobial growth inhibition (d1) from the total diameter (d2), thus AQS = (d2-d1) in mm, as described by (Zahin et al., 2010). The diameter of the clear/halo inhibition zones was interpreted as follows: Susceptible (S) ≥ 20 mm, Intermediate (I) = 15–19 mm and Resistant ≤ 14 mm, as described by the Clinical and Laboratory Standards Institute, (2020).

2.2.5.2. Quantitative antiquorum sensing assay

The microdilution method as described in section 2.2.4 was used to quantitatively evaluate the violacein reduction of plant extracts and positive control of cinnamaldehyde against *C. violaceum* ATCC 12472 (Almeida et al., 2017). Before incubation, the absorbance was read at OD_{600nm} (to check the viability and growth

of the bacterium) and OD_{485nm} (violacein production). The plates were then incubated at 30 °C for 24 h, shaking at 120 rpm. Following incubation, absorbance was read again at OD_{600nm}. Thereafter the plates were placed in a drying oven at 50 °C for 24 h. Following drying, to confirm that extracts inhibit quorum sensing with no effect on microbial growth activity, 150 µL of 100% DMSO was used to re-dissolve the dried contents in every well, intermixing and place in the shaking incubator at 30°C, 120 rpm for 1-2 hour(s). Afterwards, absorbance was read at an OD_{485nm} for violacein quantification. The percentage (%) inhibition was calculated applying equation 1:

$$\text{Percentage (\%) inhibition} = \frac{(\text{OD control} - \text{OD test})}{(\text{OD control})} \times 100 \quad (2)$$

Where OD is the optical density taken at an absorbance of 485 nm.

2.2.6. Identification of phytochemical compounds using gas chromatography-mass spectrophotometry (GC-MS)

The six plant extracts of *M. comosus* (aqueous, methanol and dichloromethane), *P. sidoides* (aqueous) and *V. karroo* (aqueous and methanol) with noticeable inhibitions were examined by GC-MS with direct injection or after silylation as trimethylsilyl derivatives of the non-volatile components. To attain the trimethylsilyl derivatives for the aqueous, dichloromethane and methanolic *M. comosus* extracts, 500 µL of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was added to the dried extracts and heated for 30 min at 70 °C. Subsequently 500 µL of pyridine was added to the reaction, filtered, and transferred to GC-MS vials. Whereas for aqueous extract of *P. sidoides* and *V. karroo* (aqueous and methanol) extracts the trimethylsilyl derivatives were acquired by adding 10 µL of N,-O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) and 10 µL of the plant sample into the GC– MS vials.

Gas chromatography-mass spectrophotometry analysis of the six extracts was carried out on a Shimadzu QP 2100 SE (Shimadzu Corporation, Tokyo, Japan) equipped with an InertCap 5MS/NP capillary (30 m × 0.25 mm × 0.25 µm: GL Sciences, Tokyo, Japan) capillary column. One µL of each sample was injected into the GC-MS in split or split-less mode depending on the concentration of the extracts. Helium was used as the carrier gas at a constant flow rate of 1.0 mL/min. The oven

temperature was programmed at 80 °C for 3 min, increased to 280 °C at 11 °C min⁻¹, and then held at this temperature for 14 min. The analysis was carried out at 70 eV in the electron impact ionisation mode. Compounds were identified tentatively based on comparison with published mass spectra libraries NIST 11 and Willey 10th edition and diagnostic ions.

2.2.7. Statistical analysis

All the experiments were done in triplicate of at least two independent experiments. The results are presented as mean $\pm$ standard deviations by Microsoft Excel. ANOVA generalised linear model (Proc GLM) was used to assess the means from inhibitory activities of extracts and controls. All statistical analyses were achieved using a Statistical Analysis System (SAS) program version 9.4, Stats Inc., 100 SAS Campus Drive, Cary NC, USA. $P < 0.05$ values were regard as statistically significant. The following criteria for antibacterial activity of extracts was used, MIC < 0.1 mg/mL (noteworthy activity), MIC 0.1 – 0.625 mg/mL (moderate activity) and MIC > 8.00 mg/mL (poor activity) (van Vuuren & Holl, 2017).

2.3. RESULTS

2.3.1. Plant extracts yields

Quantitatively, aqueous and methanolic extracts gave higher yields than ethyl acetate, acetone and dichloromethane (Table 2.1). Methanolic solvent had the highest yields from *Pelargonium sidoides* (35.9%), *Acokathera oppositifolia* (27.1%) and *Artemesia afra* (25.7%) while aqueous extracted the highest yields from *Aloe arborescens* (31.5%), *Artemesia afra* (24.3%) and *Melianthus comosus* (21.1%). *Aloe arborescens* had the lowest yields of 2.5%, 1.9% and 1.4% for acetone, dichloromethane and ethyl acetate, respectively.

Table 2.1: Plant extract yields (% w/w) obtained after extraction of medicinal plants with solvents of varying polarities.

Medicinal Plants	Solvents				
	Aqueous	Methanol	Acetone	Ethyl acetate	Dichloro-methane
<i>Acokathera oppositifolia</i>	16.2	27.1	11.5	7.2	14.9
<i>Aloe arborescens</i>	31.5	7.2	2.5	1.4	1.9
<i>Artemesia afra</i>	24.3	25.7	9.2	9.5	23.4
<i>Lippia javanica</i>	18.9	7.8	4.9	3.6	8.8
<i>Melianthus comosus</i>	21.1	22.1	6.4	3.6	2.2
<i>Pelargonium sidoides</i>	18.8	35.9	5.2	2.9	3.6
<i>Plectranthus ecklonii</i>	18.0	10.4	6.4	6.3	2.6
<i>Tetradenia riparia</i>	17.5	6.7	4.9	4.1	3.5
<i>Trichilia emetica</i>	18.9	20.0	3.6	3.9	5.3
<i>Vachellia karroo</i>	9.2	12.3	7.3	2.4	4.7

2.3.2. Minimum inhibitory concentrations (MICs) of plant extracts

The antibacterial activities of the studied plant extracts (50) against MDR pathogens (*E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus* and *S. pyogenes*) are presented as MICs (mg/mL) in Table 2.2, indicative of noteworthy or insignificant activities. The MIC values ranged from 0.19 to 6.25 mg/mL against the five tested pathogens. *Melianthus comosus* (aqueous, methanol and dichloromethane), *Pelargonium sidoides* (aqueous) and *Vachellia karroo* (aqueous and methanol) showed promising activities with an MIC value range of 0.19 - 0.78 mg/mL against the tested Gram-negative and Gram-positive bacteria. Gram-positive bacteria (GPB), *S. aureus* and *S. pyogenes* were the most vulnerable to the plant extracts: thus, potent activities were observed. Methanol extract of *M. comosus* had a MIC value of 0.19 mg/mL, aqueous and dichloromethane *M. comosus* extracts both showed MIC of 0.78 mg/mL showed significant activity while *A. afra* (acetone and ethyl acetate), *M. comosus* (acetone) and *V. karroo* (aqueous) extracts showed MIC value of 1.56 mg/mL against *S. aureus*. *M. comosus* (methanol) extract exhibited a MIC value of 0.19 mg/mL against *S. pyogenes*. Aqueous and dichloromethane *M. comosus* extracts had MICs of 0.39 mg/mL. *P. sidoides* (aqueous) extract and *V. karroo* (aqueous and methanol) extract showed the same MIC value of 0.78 mg/mL against *S. pyogenes*. The seven extracts of *M. comosus* (aqueous), *P. ecklonii* (acetone and dichloromethane), *P. sidoides* (methanol, dichloromethane), *T. riparia* (acetone) and *V. karroo* (acetone) all showed a MIC value of 1.56 mg/mL against *S. pyogenes*.

For the three GNB tested, only dichloromethane (DCM) *M. comosus* extract showed a MIC of 1.56 mg/mL against *E. coli*. Aqueous and methanolic *M. comosus* extracts both exhibited MICs of 0.78 mg/mL, whereas the *M. comosus* (DCM) extract indicated a MIC of 1.56 mg/mL against *K. pneumoniae*. *M. comosus* (dichloromethane, aqueous and methanol) extracts with a MIC value of 0.78 mg/mL showed significant activity, whereas acetone extract displayed a MIC value of 1.56 mg/mL against *P. aeruginosa*. Mostly, the extracts showed insignificant activities against GNB and GPB (Table 2.2). For all five tested bacteria, noteworthy MIC values of 0.001 mg/mL were noted for ciprofloxacin (positive control). The negative control (1% DMSO) showed no antibacterial activity against all tested strains which validated that the solvent did not affect the activity.

Table 2.2: Minimum inhibitory concentrations (mg/mL) of plant extracts tested against *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus pyogenes*

Plant extracts	<i>E. coli</i>					<i>K. pneumoniae</i>					<i>P. aeruginosa</i>					<i>S. aureus</i>					<i>S. pyogenes</i>				
	ATCC 10536					ATCC 33495					ATCC 9721					ATCC 25923					ATCC 19615				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
<i>Acokathera oppositifolia</i>	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	3.12	6.25	6.25	6.25	6.25	6.25	6.25
<i>Aloe arborescens</i>	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	3.12	6.25	6.25	3.12	3.12	3.12
<i>Artemesia afra</i>	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	3.12	3.12	3.12	6.25	3.12	3.12	1.56	1.56	6.25	6.25	3.12	3.12	3.12	3.12
<i>Lippia javanica</i>	6.25	3.12	6.25	3.12	6.25	6.25	3.12	6.25	3.12	6.25	6.25	3.12	3.12	3.12	3.12	6.25	3.12	3.12	3.12	3.12	6.25	3.12	3.12	3.12	3.12
<i>Melianthus comosus</i>	3.12	3.12	3.12	6.25	1.56	0.78	0.78	3.12	3.12	1.56	0.78	0.78	1.56	6.25	0.78	0.78	0.19	1.56	6.25	0.78	0.39	0.19	1.56	3.12	0.39
<i>Pelargonium sidoides</i>	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	3.12	6.25	3.12	6.25	6.25	6.25	6.25	0.78	1.56	6.25	3.12	1.56
<i>Plectranthus ecklonii</i>	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	3.12	6.25	6.25	6.25	6.25	6.25	1.56	6.25	3.12	1.56
<i>Tetradenia riparia</i>	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	3.12	3.12	3.12	3.12	6.25	6.25	3.12	6.25	6.25	6.25	3.12	1.56	3.12	6.25
<i>Trichilia emetica</i>	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	3.12	3.12	6.25	3.12	3.12	3.12	3.12	6.25	6.25	6.25	3.12	3.12	6.25	3.12	3.12	3.12	3.12
<i>Vachellia karroo</i>	6.25	6.25	6.25	3.12	6.25	1.56	6.25	3.12	3.12	3.12	3.12	3.12	3.12	3.12	3.12	1.56	3.12	3.12	3.12	6.25	0.78	0.78	1.56	3.12	3.12
1% DMSO	6.25					6.25					6.25					6.25					6.25				
Ciprofloxacin	0.001					0.001					0.001					0.001					0.001				

1: Aqueous; 2: Methanol; 3: Acetone; 4: Ethyl acetate and 5: Dichloromethane

2.3.3. Inhibition of quorum sensing-dependent violacein production

Chromobacterium violaceum was selected, as it is an excellent bacterium to visually detect and quantify pigment inhibition by plant extracts and phytochemicals (Chenia, 2013). The 50 extracts tested for their QS inhibition (AQS potential) qualitatively using disc diffusion at various concentrations showed that only *M. comosus* (aqueous) exhibited activity by showing opaque zones of inhibition of 13, 14 and 15 mm at concentrations of 2 x MIC, 4 x MIC and 8 x MIC (1.56 – 6.25 mg/mL), respectively. This signalled the inhibition of violacein production controlled by the QS system in *C. violaceum*. The methanolic extracts of *M. comosus* exhibited bactericidal activity, showing clear zones of inhibition of 13 mm and 15 mm at concentrations of 4 x MIC and 8 x MIC, respectively. The other extracts were not capable of reducing the violacein production of *C. violaceum*. Based on this qualitative screening, *M. comosus* (aqueous and methanol) extracts were the only extracts able to inhibit the violacein of *C. violaceum*.

Based on the qualitative violacein inhibition, some of the extracts exhibited no activity. Thus, the extracts were tested further quantitatively, to confirm their violacein inhibition by verifying the precise concentration needed for bactericidal or QS inhibition. The sub-MIC concentration (0.097 - 1.56 mg/mL; $\frac{1}{8}$ MIC - 2 x MIC) reliant on the reduction of violacein production by *C. violaceum* was shown with 8 out of 50 extracts with inhibitory effects between 2.60 - 38.34% (Figure 2.2A). The indicated moderate activity (IC_{50} values) of extracts comparable to the positive control are shown in Table 2.3.

The aqueous extract of *M. comosus* and dichloromethane extract of *P. ecklonii* exhibited violacein inhibition at 2x MIC with 38.4% (IC_{50} = 1.52 mg/mL) and 25.91% (IC_{50} = 1.81 mg/mL), respectively. *A. arborescens* (acetone and ethyl acetate), *A. afra* (dichloromethane), *M. comosus* (acetone), *P. sidoides* (aqueous) and *P. ecklonii* (methanol) presented weak AQS activity with a reduced violacein inhibition between 2 - 20%. At the MIC concentration, *M. comosus* (aqueous) and *P. ecklonii* (dichloromethane) extracts exhibited violacein inhibition of 24.33% and 24.60%, respectively while most of the extracts had less than 10% inhibition. A decrease in concentration resulted in a reduced violacein inhibitory effect at $\frac{1}{2}$ MIC, only methanolic and dichloromethane extracts of *P. ecklonii* showed 4.25% and

10.64%, respectively (Figure 2.2A). The other plant extracts have enhanced the growth of the bacterium (Figure 2.2B). The positive control, cinnamaldehyde also exhibited violacein reduction at 62.77% (MIC), 61.66% ($\frac{1}{2}$ MIC), 60.69% ($\frac{1}{4}$ MIC) and 59.43% ($\frac{1}{8}$ MIC) (Figure 2.2A). The positive control (cinnamaldehyde) slightly reduced AQS activity, as it exhibited above 40% violacein inhibition at 0.048 mg/mL with an IC_{50} value of 0.087 mg/mL.

Table 2.3: Inhibitory concentration (IC_{50}) values of the most active plant extract against *C. violaceum*.

Plant species	Half Y	IC_{50} (mg/mL)
Aqueous extract		
<i>Melianthus comosus</i>	37.66	1.52
Methanol extracts		
<i>Plectranthus ecklonii</i>	9.80	1.05
<i>Pelargonium sidoides</i>	9.70	1.45
Acetone extracts		
<i>Aloe arborescens</i>	11.51	2.49
<i>Melianthus comosus</i>	11.41	2.48
Ethyl acetate extract		
<i>Aloe arborescens</i>	12.07	1.43
Dichloromethane extracts		
<i>Artemesia afra</i>	10.23	2.24
<i>Plectranthus ecklonii</i>	27.65	1.81
Cinnamaldehyde	56.35	0.087

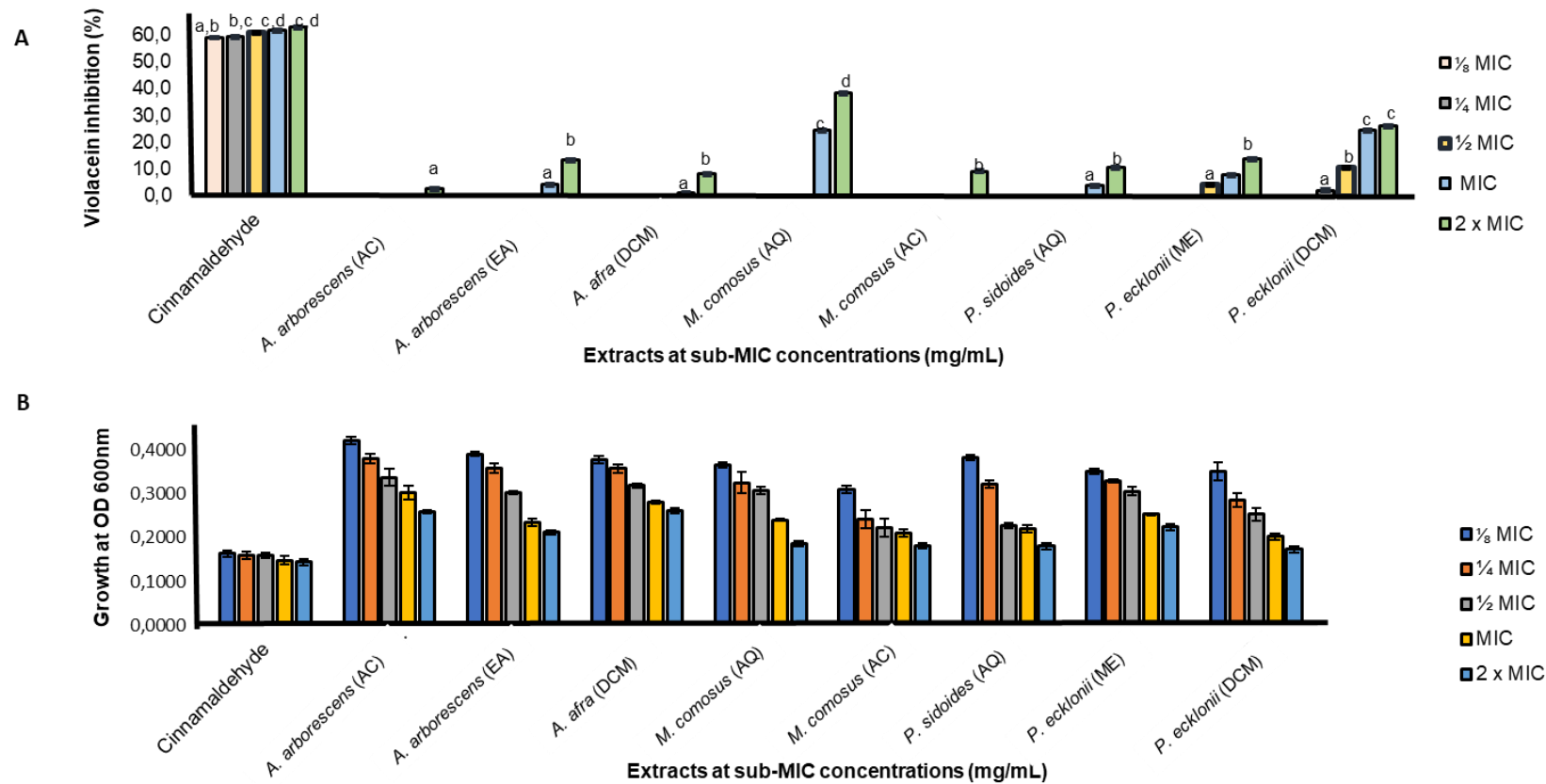


Figure 2.2A: Quantitative violacein inhibition (%) using extract against *C. violaceum* at concentrations between 0.190 -1.56 mg/mL (2 x MIC - $\frac{1}{8}$ MIC). The results are shown as the percentage of violacein inhibition. A 100% production was set as control (untreated). Statistical significance of each test across concentrations with different letters (a–d) reflects statistically significant with $p < 0.05$. **2.2B:** Growth kinetics of *C. violaceum* treated with various plant extracts at sub-MIC concentrations (indicated on the graph) after 24 hrs. Means are values of triplicate independent experiments $\pm$ SD. AQ: aqueous, ME: methanol, AC: acetone, EA: ethyl acetate and DCM: dichloromethane

2.3.4. Gas chromatographic-mass spectrometry profiling of active extracts

The chemical profiles and the identified peaks of the different plant extracts of *M. comosus* (aqueous, methanolic and dichloromethane), *P. sidoides* (aqueous) and *V. karroo* (aqueous and methanol) are shown in Figure 2.3 and Table 2.4, respectively. Chromatograms of *M. comosus* (aqueous, methanolic and dichloromethane) extracts are shown in Figure 2.3A, while *P. sidoides* (aqueous) and *V. karroo* (aqueous and methanol) extracts are shown in Figure 2.3B. These compounds belong to different chemical classes of hydrocarbons, organic acid esters, sugars, ketones, phytosteroids, acetates, and diterpene alcohols, with hydrocarbons and organic acid esters as major components (Table 2.4).

According to the library search for the identification of chemical constituents, the aqueous extract of *M. comosus* contained 12 compounds with decane, guanosine, 1,2-benzene dicarboxylic acid, diethyl ester and azelaic acid as main components (Table 2.4) and the chromatograph (Figure 2.3A). Further, 13 and 28 compounds were detected and identified in both extracts of *M. comosus* (methanol and dichloromethane), respectively, as presented in Table 2.4 and Figure 2.3A. Four main constituents were identified in *M. comosus* methanol extract as decane, guanosine, 1,2-benzene dicarboxylic acid, diethyl ester and neophytadiene. The main components detected from dichloromethane extract were heptacosane, nonacosane dotriacontane and hexatriacontane. The aqueous extract of *P. sidoides* showed eight compounds: butanoic acid, tetradecanoic acid, octadecanoic acid, octadecanoic acid, 2,3-propyl ester 2-ketobutyric acid, propanoic acid, linalool, and pyroglutamic acid. The aqueous extract of *V. karroo* comprises seven constituents: 1,2,3-propanetriol, 2-ketobutyric acid, 2-aminobutanoic acid, 1,2,3-benzenetriol, decanoic acid, hexadecanoic acid and octadecanoic acid. Methanolic extract of *V. karroo* comprised of five compounds, 1,2,3-propanetriol, α -D- glucopyranoside, methyl, hexadecanoic acid, 1-butanol, 3-methyl-, acetate and octadecanoic acid with α -D-glucopyranoside, methyl as the major constituents. The common compounds identified from these various extracts were octadecanoic acid and hexadecanoic acid

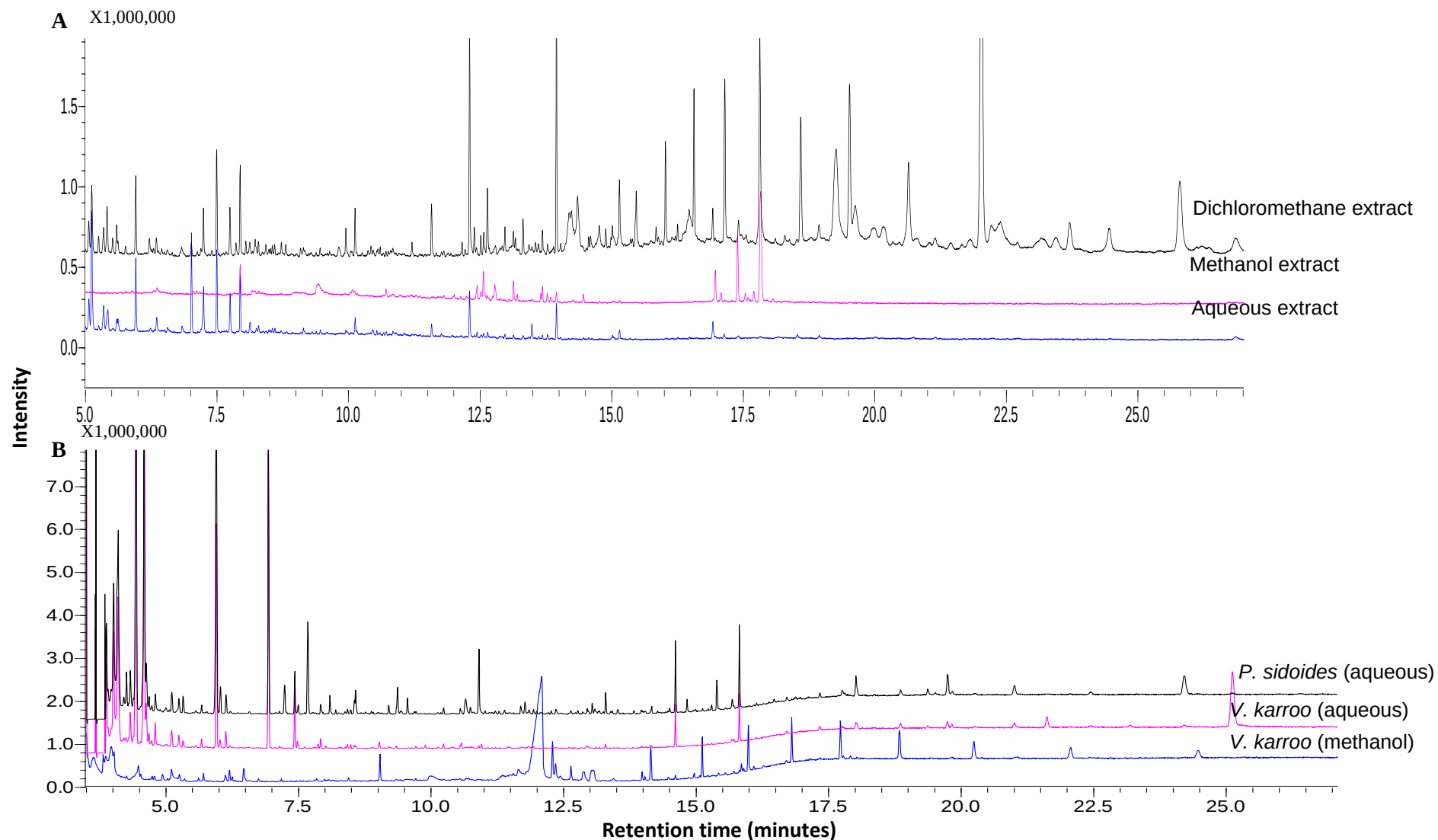


Figure 2.3: Representative total ion chromatogram (TIC) of **A:** *Melianthus comosus* (aqueous, methanol and dichloromethane) extracts and **B:** *Pelargonium sidoides* (aqueous) and *Vachellia karroo* (aqueous and methanol) extracts. All peaks' data are presented in Table 2.4

Table 2.4: Gas chromatography - mass spectrometry spectral analysis of *Melianthus comosus* (aqueous, methanol and dichloromethane), *Pelargonium sidoides* (aqueous) and *Vachellia karroo* (aqueous and methanol)

Ret. (min)	Time	Name	Molecular formula	Molecular weight	Extracts						
					<i>M. comosus</i>			<i>P. sidoides</i>		<i>V. karroo</i>	
					AQ	ME	DCM	AQ		AQ	ME
4.45		Decane	142	C ₁₀ H ₂₂	14.1%	11.3%					
4.63		1,2,3-Propanetriol	92	C ₃ H ₈ O ₃						0.8%	0.5%
5.32		Butanoic acid*	160	C ₇ H ₁₆ O ₂ Si				0.4%			
6.02		Propanoic acid*	234	C ₉ H ₂₂ O ₃ Si ₂		1.4		0.8%			
6.43		1-Butanol, 3-methyl-, acetate	130	C ₇ H ₁₄ O ₂							1.2%
		2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	144	C ₆ H ₈ O ₄	5.0%						
6.48		2-Ketobutyric acid eo-tms*	217	C ₉ H ₁₉ NO ₃ Si				1.0%		1.4%	
7.42		Cyclooctane*	112	C ₈ H ₁₆		1.2%					
7.81		2-Aminobutanoic acid,bis-TMS*	247	C ₁₀ H ₂₅ NO ₂ Si ₂						0.2%	
7.91		Guanosine	283	C ₁₀ H ₁₃ N ₄ O	9.8%	22.3%					
9.39		1,2,3-Benzenetriol	126	C ₆ H ₆ O ₃						0.3%	
9.51		Decanoic acid*	244	C ₁₃ H ₂₈ O ₂ Si						0.1%	
10.23		Linalool	154	C ₁₀ H ₁₈ O				3.2%			
10.43		1,2-Benzenedicarboxylic acid, diethyl ester	236	C ₁₂ H ₁₄ NO ₆	64.1%	52.8%	2.0%				
10.84		Pyroglutamic acid	129	C ₅ H ₇ NO ₃				1.7%			
10.90		Octanedioic acid*	318	C ₁₄ H ₃₀ O ₄ Si ₂	1.2%		1.2%				
11.58		α-D-Glucopyranoside, methyl	194	C ₇ H ₁₄ O ₆							44%
12.09		(-)-Loliolide	196	C ₁₁ H ₁₆ O ₃			0.2%				
12.27		Azelaic acid*	332	C ₁₅ H ₃₂ O ₄ Si ₂	4.8%		5.6%				
12.30		D-Galactose, 2,3,4,5,6-pentakis-O*	570	C ₂₂ H ₅₅ O ₆ Si ₅	0.4%	2.5%					
12.44		D- (-)-Fructofuranose, pentakis ether*	541	C ₂₁ H ₅₂ O ₆ Si ₅		2.0%	0.5%				
12.51		Neophytadiene	278	C ₂₀ H ₃₈	2.4%	5.4%	0.7%				
12.60		Tetradecanoic acid*	300	C ₁₇ H ₃₆ O ₂ Si			1.4%	0.5%			
12.64		Cyclopentadecanol	226	C ₁₅ H ₃₀ O		2.2%	0.4%				
12.89		Decanedioic acid*	346	C ₁₆ H ₃₄ O ₄ Si ₂			0.4%				
12.94		Benzoic acid 3,4,5-tris(trimethylsiloxy)*	458	C ₁₉ H ₃₈ O ₅ Si ₄	1.5%						
13.48		Hexadecanoic acid*	328	C ₁₉ H ₄₀ O ₂ Si	3.4%	1.4%	5.4%			1.0%	0.6%
13.95		Tetramethyl hexadecenol (Trans phytol)	296	C ₂₀ H ₄₀ O		4.8%	0.9%				
14.41		Docosane	310	C ₂₂ H ₄₆			0.4%				

Table 2.4 Continued.

15.12	Octadecanoic acid*	356	C ₂₁ H ₄₄ O ₂ Si		1.4%	1.9%	1.1%	0.5%
15.47	Tricosane	324	C ₂₃ H ₄₈		1.2%			
16.02	Tetracosane	338	C ₂₄ H ₅₀		2.5%			
16.56	Pentacosane	352	C ₂₅ H ₅₂		4.2%			
16.92	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	390	C ₂₄ H ₃₈ O ₄	1.9%	0.7%			
16.97	Sucrose	342	C ₁₂ H ₂₂ O ₁₁		4.8%			
17.15	Hexacosane	366	C ₂₆ H ₅₄		5.7%			
17.70	Thymol-. beta. -d-glucopyranoside, tetrakis*	600	C ₂₈ H ₅₆ O ₆ Si ₄		2.2%			
17.81	Heptacosane	380	C ₂₇ H ₅₆		7.0%			
17.81	Tetratetracontane*	618	C ₄₄ H ₉₀		6.9%			
18.58	Octacosane	394	C ₂₈ H ₅₈		6.4%			
19.50	Nonacosane	408	C ₂₉ H ₆₀		9.6%			
19.26	1-Hentetracontanol	592	C ₄₁ H ₈₄ O		7.2%			
19.74	Octadecanoic acid, 2,3-propyl ester*	502	C ₂₇ H ₅₈ O ₄ Si ₂			0.9%		
20.62	Triaccontane	422	C ₃₀ H ₆₂		5.0%			
22.00	Dotriacontane	450	C ₃₂ H ₆₆	4.6%	33.9%			
22.03	Hexatraiccontane*	506	C ₃₆ H ₇₄		21.6%			
22.86	alpha-Tocopherol (Vitamin E)	430	C ₂₉ H ₅₀ O ₂		1.6%			
23.66	Tetratricontane	478	C ₃₄ H ₇₀		3.0%			

*Indicates compounds that are derivatives

2.4. DISCUSSION

For ancient years, communities have been using natural products for treating infections, as the indigenous plants provide bioactive phytochemicals that lead to the development of novel therapeutics (Anand et al., 2019).

The extraction process is vital for sourcing various bioactive phytochemicals from medicinal plants: hence, we used solvents of different polarities. This is further substantiated by the fact that the solubility of plant extracts differs per solvent and relative to the plant part used for a variety of phytoconstituents. Here, aqueous and methanol extracts have higher yields than ethyl acetate, dichloromethane and acetone. Similar to our study, Mabona et al. (2013); Eloff, Angeh & McGaw (2018) indicated that methanol and water had higher percentage extraction yields. Thus, larger yields do not mean better biological activity, as there are different proportions of compounds present in a plant.

Traditional healers and the community of South Africa at large often use medicinal plants as an alternative herbal medicine for infectious diseases (Semenya & Maroyi, 2013). This has led to researchers studying plants for their antimicrobial properties, specifically with the reference strains, which more or less corresponds with the literature (Elisha et al., 2017; van Vuuren & Muhlarhi, 2017; Shirinda et al., 2019). The efficiency of plant extracts as potential antibacterials may be attributed to plants continuously protecting themselves against phytopathogens that are within their environment. This triggers the production of broad-spectrum compounds with antimicrobial activity (Nguta & Kiraithe, 2019).

To authenticate the antibacterial activities of 10 medicinal plants, a noteworthy activity of a plant extract was regarded when the MIC value was 1 mg/mL or lower (van Vuuren & Muhlarhi, 2017). Susceptibility against *S. aureus*, *P. aeruginosa*, *E. coli*, *S. pyogenes* and *K. pneumoniae* was observed for *Artemisia afra* (acetone & ethyl acetate), *M. comosus* (aqueous, methanol, acetone and dichloromethane), *T. riparia* (acetone), *P. ecklonii* (methanol & dichloromethane), *P. sidoides* (aqueous, methanol & dichloromethane) and *V. karroo* (aqueous, methanol & acetone) extracts showing noteworthy MIC values (Table 2.2). Compared to our study, van Vuuren & Muhlarhi (2017) reported higher MIC values (>8.00 mg/mL) for aqueous extracts of *A. afra*, *L. javanica* and *T. riparia* against *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *S. aureus*. Our study also reported improved MIC values of 6.25

mg/mL for aqueous extracts of *A. afra*, *L. javanica* and *T. riparia* against the four tested pathogens. Mulaudzi et al. (2011) indicated a significant MIC value of 0.195 mg/mL for *V. karroo* (aqueous) extract against *S. aureus*, while our study showed an aqueous extract of *V. karroo* had a MIC value of 1.56 mg/mL against *S. aureus*.

Incongruent with the current study, Aboobaker et al. (2019) reported weak activities between 1.00 and 4.00 mg/mL of aqueous and organic extracts of *P. sidoides* whereas our study reported an MIC value of 6.25 mg/mL for *P. sidoides* (aqueous and organic) extracts against *S. aureus*. Based on our results, an aqueous extract of *P. sidoides* exhibited good activity (0.78 mg/mL) against *S. pyogenes*. Contrary to our findings, Kelmanson, Jager & Staden (2000) reported no bacterial activity of the *M. comosus* (methanol) extract against *P. aeruginosa* and *E. coli*, while only *S. aureus* showed a MIC value of 2.00 mg/mL, whereas Mabona et al. (2013) reported MIC values of 1.60 and 2.00 mg/mL against *S. aureus* and *P. aeruginosa*, respectively. The most effective plant species indicating significant activity against the resistant strains was *M. comosus*, followed by *V. karroo* > *P. sidoides* > *P. ecklonni* and *A. afra*. According to van Vuuren & Muhlarhi (2017); Mamabolo et al. (2018) plant extracts showing MIC values < 1 mg/mL, are regarded as noteworthy. On that basis, our extracts exhibited good activities and could be promising natural antibacterials for the studied test pathogens.

Generally, GPB (*S. aureus* and *S. pyogenes*) are more susceptible to the treatment of plant extracts than GNB (*E. coli*, *K. pneumoniae* and *P. aeruginosa*) which demonstrate moderate activities. This is attributed to the inability of plant extracts to penetrate through the outer membrane (OM) of GNB due to the structural lipopolysaccharide component (De Zoysa et al., 2019). No antibacterial activity does not signify inactive bioactive compounds within the plants, instead, inadequate quantities of phytochemicals in the extract could be the reason for poor activity (De Zoysa et al., 2019). The results obtained in this study confirmed the effective use of these extracts: thus, this instigated a further investigation of the antipathogenic properties of the extracts.

As mentioned above, plant extracts continuously demonstrate bactericidal activity; however, few reports have explored their ability to interfere with the QS signaling mechanism. Targeting QS signaling emerges as an innovative strategy, as this mechanism focuses on virulence factors such as biofilms and others, tackling pathogenic bacteria and thereby disarming them (Yüzbaşıoğlu et al., 2017). A

previous study by Alreshidi et al. (2020) showed that using the disc diffusion assay, the *T. polium* methanolic extract had no inhibitory effects against *C. violaceum*. Based on the above results (Figure 2.2A), plant extracts have some inhibitory effect at a higher concentration (2 X MIC) while at a lower concentration ($\frac{1}{8}$ MIC) there were insignificant inhibitory effects of plant extracts on violacein production. Comparable to our study, Yüzbaşıoğlu et al. (2017) documented plant extracts of Turkey origin to slightly reduce the violacein production of *C. violaceum* ATCC 12472 at a sub-MIC concentration (0.25 mg/mL). Bodede et al. (2018) reported a 14.2 - 28% quorum sensing inhibition (QSI) of six extracts of *S. nigrescens* at a concentration of 0.6 mg/mL. These findings validate that plant extracts possess AQS/QSI activities at sublethal concentrations. Vatterm et al. (2007) mentioned that natural products or extracts inhibit the accumulation of QS signals by reducing the concentration of active signal molecules either enzymatically or nonenzymatically. Furthermore, this can also be accomplished by phytochemicals competitively or noncompetitively binding to the receptor protein.

Thus far, research studies have made it completely clear that natural products, as well as medicinal plants, are a promising source of AQS/QSI compounds. The present study revealed that the leaf parts of *M. comosus*, *P. ecklonii* and *P. sidoides* exert bacteriostatic and/or antipathogenic activities against *C. violaceum* which may promote the possibility of the plant's defence mechanism against diverse bacterial invasions. Notably, the plant extracts of *V. karroo* exhibited significant antibacterial activities against GNB and GPB but did not show any AQS activities: thus, these plant extracts could exhibit good potency against MDR bacteria through different mechanisms of action.

Because *M. comosus*, *P. sidoides* and *V. karroo* extracts were most potent in inhibiting bacterial growth of GNB and GPB and potentially inhibited QS-dependent violacein production, the plants were then chosen for further analysis of the chemical compounds using GC–MS.

The profiles confirm the presence of phytoconstituents of pharmaceutical value and provide a better understanding of the nature of the medicinal properties of the plants (Adonizio et al., 2006). In this study, the GC–MS method was chosen to distinguish the phytoconstituents found in *M. comosus*, *P. sidoides* and *V. karroo* and revealed common saturated fatty acids of octadecanoic acid and hexadecanoic acids which are well-known as stearic acid and palmitic acid, respectively. These

bioactive compounds are described to have antibacterial activity against *P. aeruginosa* and *S. aureus* (Ivanova et al., 2017).

The chemical profiles of *M. comosus*, *P. sidoides* and *V. karroo* extracts confirmed the presence of phytoconstituents of pharmaceutical value and provided an improved understanding of the nature of the medicinal properties of the plants. The GC-MS analysis (Figure 2.3A) of *M. comosus* (aqueous, methanol and dichloromethane) revealed various classes of compounds such as flavonoids, phytosterols and terpenoids that possess biological activities which have a defence mechanism against pathogenic *S. aureus*, *E. coli*, *K. pneumonia* and *P. aeruginosa* (Eloff, Angeh & Mcgaw, 2018). Specifically, the phytosteroid 3 β -stigmast-5-en-3-ol possesses antibacterial activity against *S. aureus* and *E. coli* (Ododo, Choudhury & Dekebo, 2016). Gólezbiowski et al. (2013) have reported the antibacterial activity of azelaic acid and decanedioic acid against *Staphylococcus* species and *Bacillus subtilis*. Based on the chemical profiles of these extracts, it is feasible to explore the QS mechanisms using virtual molecular docking screening to better understand the network of multiple responses prompted by AHLs (Ahmad, Viljoen & Chenia, 2014).

Chemical characterization of *P. sidoides* has shown that the plant has a rich source of tannins, fatty acids, phenolics, coumarins and flavonoids (Kolodziej, 2007). Several studies by Nyila et al. (2010); Priyanka et al. (2015); Maroyi (2017b) reported phytochemical analysis of various plant parts of *V. karroo*, revealing various classes of compounds, such as terpenes, tannins, phenols, proanthocyanidin and flavonoids. Priyanka et al. (2015) identified various constituents of compounds from the roots and leaves of *V. karroo* (chloroform, ethanol, and ethyl acetate) using GC–MS analysis. Several efficient methods for the identification of bioactive compounds are GC–MS which can detect diverse classes of compounds, such as steroids, alkaloids, organic acids, esters, hydrocarbons, amino acids and alcohols (Konappa et al., 2020). Several of these identified compounds have been shown to exert antibacterial activities against various pathogens indicating that they could be responsible for the biological activities of the extracts. However, to comprehend their mode of action, an analysis of the compound's molecular mechanism is necessary.

2.5. CONCLUSION

The findings of the study confirmed the antibacterial activity of *A. afra*, *P. ecklonii*, *P. sidoides*, *V. karoo* and *M. comosus* extracts. It further highlighted the significance of assessing the unexplored AQS properties of medicinal plants. The plant extracts have shown the potential to disrupt the violacein production of *C. violaceum*. However, *M. comosus*, *P. sidoides* and *V. karoo* were the most potent plants and the focus will be on the bioactive components of these plants. This study will further present the significance of the molecular docking strategy in the discovery of novel compounds. The significance of molecular mechanisms underlying the biological activity gives a clear understanding and insight, especially if the compounds are to be further studied for potential applications. Therefore, the molecular mechanisms of the specific compounds on their quorum sensing inhibition are reported in the next chapter.

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CHAPTER THREE

IN-SILICO VALIDATION OF ANTIQUORUM SENSING ACTIVITIES VIA CVIR' PROTEIN HOMOLOGUE IN CHROMOBACTERIUM VIOLACEUM

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3.1. INTRODUCTION

The increasing awareness regarding the relationship between quorum sensing (QS) bacterial pathogenicity and antimicrobial action has led the scientific community to focus on compounds that have the potential to disrupt the mechanism of QS (Singh & Bhatia, 2018). This advanced approach to preventing or disrupting QS is growing impetus (Qin et al., 2020). This is because QS plays an imperative role in bacterial cellular functions and survival tactics. Thus, presenting blocking the QS signal production or reception as one of the promising strategies which potentially can impede the biofilms and other virulence factors; ideally to combat or snag the multidrug-resistant (MDR) challenge (Alvarez et al., 2014; Aliyu et al., 2016).

The bacterial QS interaction depends on the small, secreted signalling molecules. In most GNB, QS systems utilise these AHL signal molecules. Descriptively, the AHL molecular structure possesses (i) a homoserine lactone ring and (ii) an acyl chain bonded with an amide, (Ahmad, Viljoen & Chenia, 2014). Because these are implicated in triggering pathogenicity and virulence genes, this sets them as novel targets for unique mechanisms of action of antimicrobials, now greatly studied as antipathogenic agents (Alvarez et al., 2014). The compounds that act as QS antagonists by blocking signal molecules may be advantageous not only to remodel plant-derived molecular mixtures but for the development of novel drugs (Deryabin et al., 2019). For example, compounds like coumarins, terpenoids, benzoic acid derivatives, flavonoids and tannins possess non-competitive or competitive inhibitory potential by binding to the active site of the AHL (Paul, Guzman & Padilla, 2017).

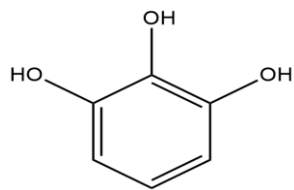
Hence, this study selected the *Chromobacterium violaceum* strain as a model organism to investigate and validate that the selected identified compounds from the active extracts are responsible for the QS inhibitory potential, relative to the CviR (receptor) protein. *C. violaceum* QS system entails (i) Cvil/CviR, a LuxI/LuxR homologue, and (ii) autoinducer molecules such as C8-AHL and N-decanoyl-homoserine lactone (C10-HSL), while N-hexanoyl-L-acyl homoserine lactone (C6-AHL) which strongly antagonize the receptor or acts as a partial antagonist to activate the expression of violacein production (Chenia, 2013; Lasarre & Federle, 2013).

Here, computer-based tools were employed for the virtual evaluation of potential QS inhibitors from the plant's bioactive compounds. This application was hypothesized to improve the insights into the molecule's mechanism bioactivity, contributing to the quest for potential novel antipathogenic drug candidates (Macalino et al., 2015). Furthermore, *in-silico* molecular docking (MD) and molecular simulations (MS) were used in the validation of the compounds from active plant extracts as potential drivers of the QSI activity, evaluated *in vitro*. This was done to comprehend the binding affinities of the CviR'-binding pocket as a QSI model target on selected phytochemical compounds from *Melianthus comosus*, *Pelargonium sidoides* and *Vachellia karroo* (active plants, *in vitro*) as little is known about these plant's phytochemicals molecular modelling mechanism of action against bacterial QS receptor proteins.

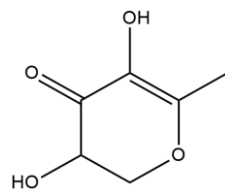
3.2. MATERIALS AND METHODS

3.2.1. Selection of suitable compounds as antagonists of CViR protein homologue: 3QP1

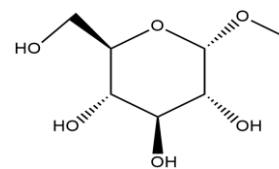
The following criterion formed the basis of selecting QS antagonistic compounds (i) structural similarity to the signal molecules; (ii) low molecular weight; and (iii) having a hydroxyl group if it is a side chain compound. Furthermore, the compounds may possess a five-membered lactone ring with an acyl group as a spacer and hydrophobic tail which enables binding to the active site by hydrogen bonding and hydrophobic interactions (Aliyu et al., 2016). These QSIs can structurally be similar to the natural ligand or conversely, can have an utterly diverse molecular structure (Qin et al., 2020). For these reasons, the following phytochemical compounds shown in Figure 3.1, identified (as per the previous chapter) from *M. comosus*, *P. sidoides* and *V. karroo* extracts were selected for molecular docking studies based on the criteria and their chemical structures.



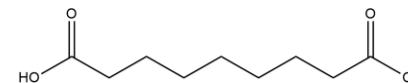
1,2,3-Benzenetriol



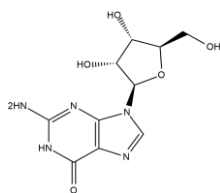
2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one



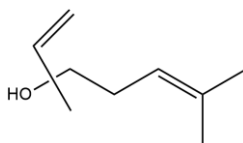
α -D-glucopyranoside, methyl



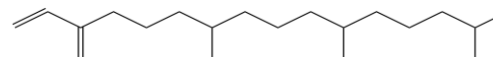
Decanedioic acid



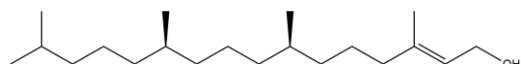
Guanosine



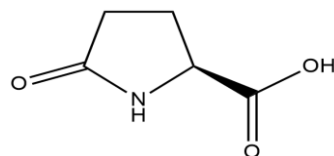
Linalool



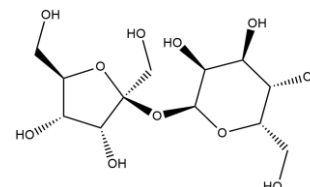
Neophytadiene



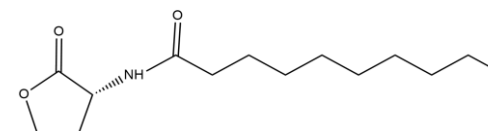
Phytol



Pyroglutamic acid



Sucrose



C10-homoserine lactone

Figure 3.1: Chemical structures of selected compounds as AHL analogues or non-AHL-like molecules with specific agonist or antagonist activity.

3.2.2. Molecular docking studies of selected phytochemicals

Molecular docking analysis was conducted to explore the inhibitory mechanism and the binding interactions of selected compounds against the CviR' protein of *C. violaceum* (PDB: 3QP1) as described by Cosa et al. (2020). The 2-dimensional structures of the 10 selected phytochemicals were obtained from the chemical library PubChem and drawn on Canvas 3.5 and exported to Maestro 11.5. The 3QP1 crystallized structure with the native ligand (C10-HSL), was obtained from the Protein Data Bank database (PDB). Before the docking experiments, chemically correct models of the ligands were generated using the LigPrep of Schrodinger, and the receptor structure through a protein preparation wizard. Thereafter, docking was done using the Glide ligand docking module and Glide receptor for the grids. All docking calculations were performed using AutoDock 4.0 and Grids (Schrodinger, LLC, New York, NY, USA) for the prepared protein generated using the protein grid generation module. Further modifications included the removal of water and metals before optimizing the hydrogen bonds thus forcing minimization resulting in the generated scores mimicking the potential energy change when the protein and the compound come together based on hydrogen. A cut-off docking score between -5 and -15 kcal/mol was established to differentiate between potential active and inactive compounds (Pereira et al., 2019).

3.2.3. Molecular dynamics simulations for the best-docked ligands

Molecular dynamic simulations (MDS) were conducted to evaluate the stability of the protein-ligand structure. Apo, a protein without a ligand was used to compare with the protein-ligand complexes to observe any protein dynamics changes. The best-docked ligands included α -D-glucopyranoside, methyl, 1,2,3-benzenetriol, guanosine, phytol and sucrose. The reference compounds, cinnamaldehyde and quercetin were also examined further for MDS analysis against the target protein 3QP1. The systems were prepared for MD simulation comprising each of the 6 phytochemical compounds bound to the 3QP1 and the unbound states of the 3QP1 protein target (Apo). Molecular dynamic simulations were implemented using the AMBER 18 suite with an integrated PMEMD module engine with a Graphic Processing Unit (GPU). The antechamber phase of AMBER 18 was used to

parameterize the ligands, while the 3QP1 protein was parameterized with the AMBER force field, FF14SB (Salomon-Ferrer et al., 2013; Ganesan, Coote & Barakat, 2017; Raabe, 2017). The LEAP module incorporated in the AMBER 18 suite was used to neutralize and solvate the systems by adding counter ions such as chloride and sodium ions, thereafter, suspending them in an orthorhombic box of TIP3P water molecules such that all atoms are enclosed within 8 Å of the box edges. The amino acid residues of the protein were renumbered due to missing residues in the initial crystal structure. The initial energy minimization of 2500 steps was performed with a restraint potential of 10 kcal/mol⁻¹ Å⁻² applied to the solutes, for 1000 steps of steepest descent followed by 1000 steps of conjugate gradient minimization. An additional unrestrained full minimization of 200 steps was performed by conjugate gradient algorithm. The systems were gradually heated using a Langevin thermostat in a canonical ensemble (NVT) from 0 – 300 K. Thereafter, each system was equilibrated for 500 ps with a constant operating temperature of 300 K, as well as a constant pressure of 1 bar using the Berendsen barostat and number of atoms resembling an isobaric-isothermal ensemble. The overall simulation was conducted for 100 ns, where each simulation incorporated the SHAKE algorithm to restrain hydrogen bonds.

3.2.4. Post-molecular dynamic analysis

Following the simulation run, coordinates of all systems (unbound and bound) were saved at 1ps intervals followed by a subsequent analysis of trajectories using the integrated PTRAJ and CPPTRAJ module (Roe & Cheatham, 2013). Visualization of the complexes and data plots were carried out using the graphical user interface of UCSF chimera (Pettersen et al., 2004), Discovery studio (BIOVIA, 2016) and Microcal Origin analytical software (Seifert, 2014). Root-mean-square deviation (RMSD) and root-mean-square-fluctuation (RMSF) analyses were used to evaluate the stability and flexibility of the potential QS antagonistic compounds and the QS protein targets. BIOVIA Discovery Studio visualization software was employed to visualize all crucial molecular interactions during the simulation.

3.2.5. Computation of thermodynamic binding free energy

The molecular mechanics with generalized born and surface area solvation (MM/GBSA) method was utilized to determine the binding free energy (ΔG_{bind}) for the following ligands of 1,2,3-benzenetriol, α -D-glucopyranoside, methyl, guanosine, phytol, sucrose, cinnamaldehyde and quercetin to biological macromolecules (Genheden & Ryde, 2015). This method is generally well known for assessing the binding energetics of molecular dynamics simulated systems of protein-ligand complexes through precision calculations (Zhang, Pérez-Sánchez & Lightstone, 2015). Mathematically, binding free energy computation can be represented as follows:

$$(1) \Delta G_{\text{bind}} = G_{\text{complex}} - G_{\text{receptor}} - G_{\text{ligand}}$$

$$(2) \Delta G_{\text{bind}} = E_{\text{gas}} + G_{\text{sol}} - TS$$

$$(3) E_{\text{gas}} = E_{\text{int}} + E_{\text{vdW}} + E_{\text{ele}}$$

$$(4) G_{\text{sol}} = G_{\text{GB}} + G_{\text{SA}}$$

$$(5) G_{\text{SA}} = \gamma \text{SASA}$$

Where:

E_{ele}	Electrostatic potential energy from Coulomb forces
E_{gas}	Gas-phase energy (based on FF14SB force field terms)
E_{int}	Internal energy
E_{vdw}	van der Waals energy
G_{sol}	Solvation free energy
G_{GB}	Polar solvation energy
G_{SA}	non-polar solvation energy
S	Total entropy of solute
T	Total entropy of temperature

3.3. RESULTS

3.3.1. *In-silico* molecular docking of phytochemical compounds

When MD was performed to investigate the binding potential and interactions of the phytochemical compounds in QS receptor: CviR' (PDB ID: 3QP1) protein, only five compounds: sucrose, decanedioic acid, α -D-glucopyranoside, methyl, guanosine and 1,2,3-benzenetriol showed good binding affinities, whereas other compounds showed weak binding affinities and the remaining compounds were incapable of binding to the receptor protein. The data presented in Table 3.1 summarises the docking scores, glide energy and hydrogen bonds while Figure 3.2 shows the ligand interaction for only the best docked ligand-3QP1 protein complex. Quercetin, cinnamaldehyde and C10-homoserine lactone were used as QSI reference compounds to ascertain the binding mechanism with the QS proteins.

Molecular docking of the five of the selected compounds identified from *M. comosus*, *P. sidoides* and *V. karroo* extracts, showed that sucrose was the most active potential QS antagonistic compound with a docking score of -7.59 kcal/mol and glide energy of -32.20 kcal/mol, forming hydrogen bonds with amino acids: Tyr80, Trp84 and Asp97 and sixteen hydrophobic interactions (Figure 3.2A). Decanedioic acid had a docking score of -6.99 kcal/mol with glide energy of -39.29 kcal/mol, formed hydrogen bonds with Tyr80 and Ser155 and eighteen hydrophobic interactions (Figure 3.2B). Guanosine showed a docking score of -5.96 kcal/mol with glide energy of -25.64 kcal/mol, followed by α -D-glucopyranoside, methyl with a docking score of -5.90 kcal/mol and glide energy of -30.09 kcal/mol then 1,2,3-benzenetriol showed a docking score of -5.57 kcal/mol with glide energy of -23.61 kcal/mol. Guanosine was shown to have 18 hydrophobic interactions and formed hydrogen bonds with Trp84 and Met135 (Figure 3.2C), followed by α -D-glucopyranoside, methyl forming 13 hydrophobic interactions and binding with hydrogen bonding to Trp84 and Met135 (Figure 3.2D) while 1,2,3-benzenetriol formed 13 hydrophobic interactions, hydrogen bonding with Tyr80, Trp84 and Ser155 and π - π staking interaction with Tyr80 (Figure 3.2E). Compounds of 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one, linalool, neophytadiene, phytol and pyroglutamic acid showed less than -5.00 kcal/mol indicating weaker docking scores with the 3QP1 protein.

The native ligand of C10-homoserine lactone showed a -7.61 kcal/mol with glide energy of -46.99 kcal/mol binding with hydrogen bonding to Tyr80, Trp84 and Ser155 (Figure 3.2F) whereas the positive controls of quercetin and cinnamaldehyde showed docking scores of -10.613 and -5.949 kcal/mol, respectively. About eighteen hydrophobic interactions were observed for quercetin with hydrogen bond interactions to Ser155, Asp97, Met135, π - π staking with Tyr80 and Tyr 88 (Figure 3.2G) while less binding, amino acid interaction (Trp84, only) and π - π staking with Tyr 88 was observed for cinnamaldehyde (Figure 3.2H).

Table 3.1: Docking results of the selected compounds on 3QP1

Compound name	Docking score	Glide energy	Interactions
α -D-glucopyranoside, methyl	-5.90	-30.09	Trp84, Met135
1,2,3-Benzenetriol	-5.57	-23.61	Tyr80, Trp84, Ser155
2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	-4.97	-27.72	Tyr80, Ser155
Decanedioic acid	-6.99	-39.29	Tyr80, Ser155
Guanosine	-5.96	-25.64	Trp84, Met135
Linalool	-4.98	-21.56	Tyr80
Neophytadiene	-4.26	-28.72	Nil
Phytol	-4.42	-19.20	Val75
Pyroglutamic acid	-3.88	-26.144	Tyr80, Trp84, Ser155
Sucrose	-7.59	-32.20	Tyr80, Trp84, Asp97
Reference compounds			
C10-HSL	-7.61	-46.99	Tyr80, Trp84, Ser155
Cinnamaldehyde	-5.94	-24.20	Trp84
Quercetin	-10.61	-44.87	Met 135, Ser155, Asp97

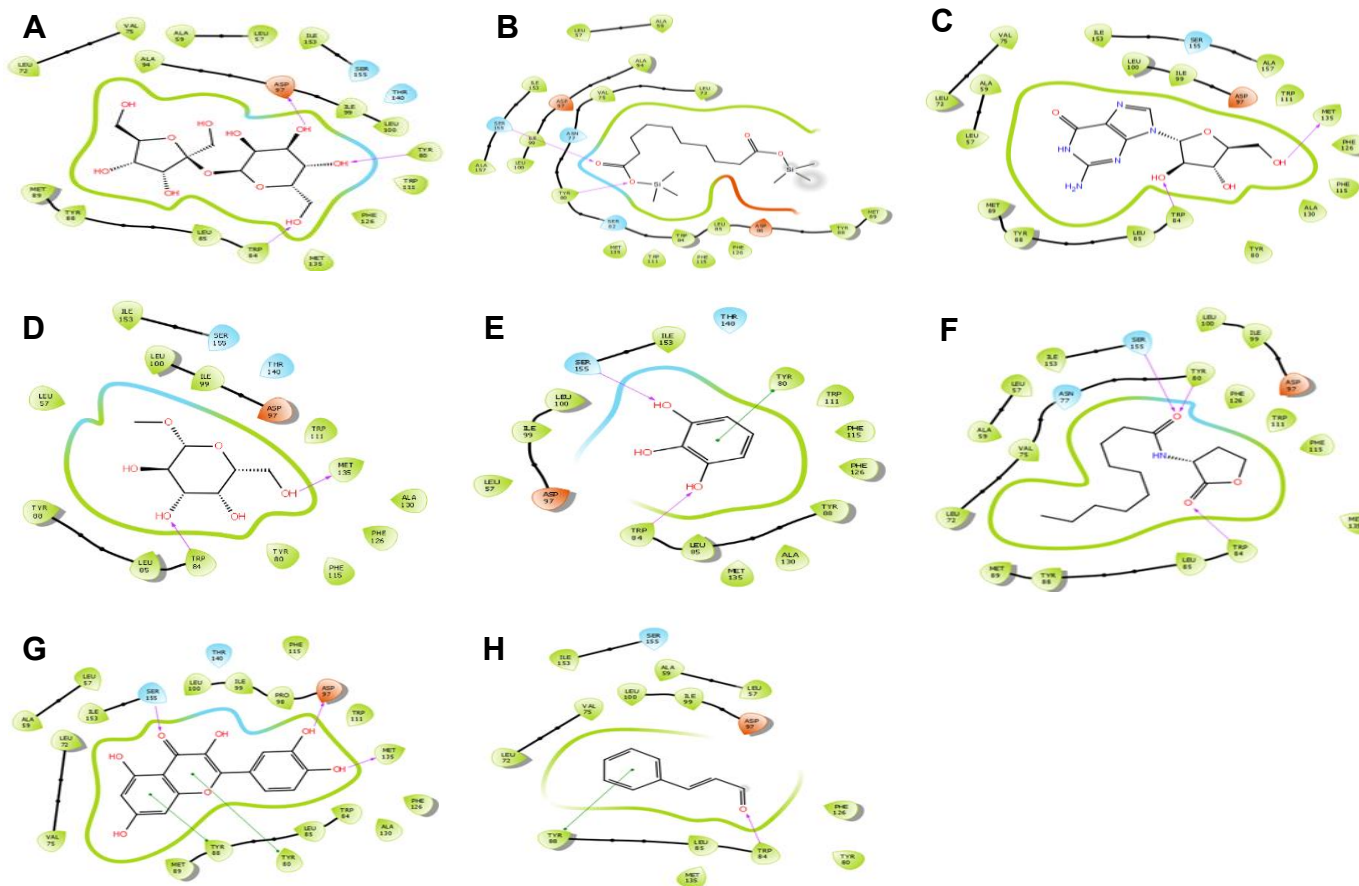


Figure 3.2: Interaction network between best-docked compounds and 3QP1 protein. The protein residues with a negative charge are shown in red, positive charge in velvet, polar in cyan, and hydrophobic in parrot green. The H-bond interactions are shown as a purple arrow, pi-pi stacking as a green line. (A) Sucrose; (B) Decanedioic acid; (C) Guanosine; (D) α -D-Glucopyranoside, methyl; (E) 1,2,3-Benzenetriol; (F) C10-homoserine lactone; (G) Quercetin and (H) Cinnamaldehyde.

3.3.2. Molecular dynamics simulations

3.3.2.1. Structural stability and flexibility of 3QP1 protein

When the best-docked complexes were evaluated for structural analysis, calculating the RMSD of each pose against its conformation in the crystal structure, the overall protein convergence and MD stability trajectories were determined as shown in Figure 3.3. The following colours are a representation of each compound, Apo (black), 1,2,3-benzenetriol (red), α -D-glucopyranoside, methyl (green), guanosine (blue), phytol (cyan), sucrose (purple), cinnamaldehyde (yellow) and quercetin (mustard). In the 3QP1 system, the convergence was achieved early in the simulation at around 5 ns where atomic motions moved steadily. There was a gradual separation of atomic motions in the 3QP1 bound by the guanosine (blue) system after about 10 ns (Figure 3.3A). Peak separation was observed around the 15 ns mark which was characterized by higher/increased atomic deviations which continued to the 60 ns mark and appear to move steadily until the end of the simulation. The average RMSD values estimated for all systems were 1.64Å, 1.81Å, 1.61Å, 3.00Å, 1.68Å, 1.98Å, 1.93Å and 1.67Å for the Apo, 1,2,3-benzenetriol, α -D-glucopyranoside, methyl, guanosine, phytol, sucrose, cinnamaldehyde and quercetin systems, respectively.

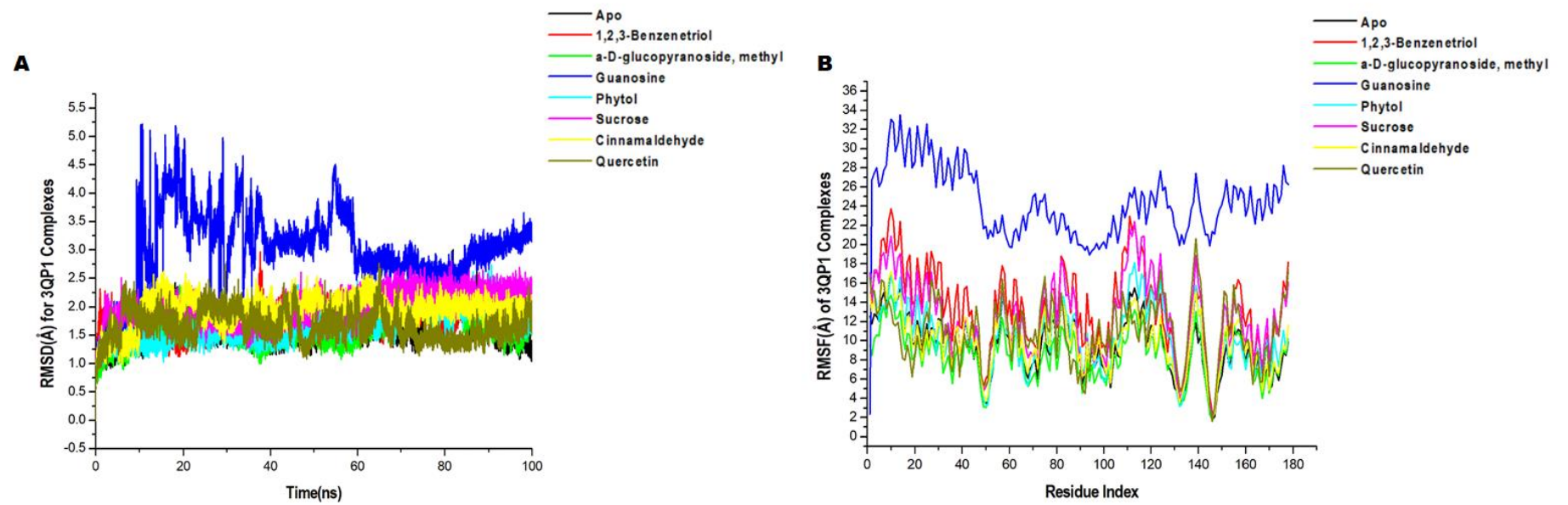


Figure 3.3: (A) C- α root-mean-square deviation (RMSD) and (B) C- α root-mean-square-fluctuation (RMSF) plots of unbound and bound quorum sensing protein 3QP1 complex with the phytochemical compounds during the 100 ns simulation.

The effect of the RMSF analysis approach was utilized in determining the changes in residual motions as a measure of the flexibility of certain regions of the 3QP1 structure. The RMSF averages computed for all systems were 9.36Å, 13.33Å, 8.39Å, 24.35Å, 10.25Å, 12.51Å, 9.93Å, and 10.98Å for the Apo, 1,2,3-benzenetriol, α -D-glucopyranoside, methyl, guanosine, phytol, sucrose, cinnamaldehyde and quercetin, respectively (Figure 3.3B). All systems showed to have a higher RMSF average as compared to the Apo (black) system except for the α -D-glucopyranoside, methyl (green) bound system which appeared to have lower fluctuation of c- α atoms suggesting a less flexible structure.

3.3.2.2. Differential binding free energy analysis

After establishing the impact of the phytochemicals on the conformational dynamics of the selected QS protein targets, a further approximation of the thermodynamics of the complexes was determined through the MM/GBSA method. The binding affinity is indicative of the strength of interactions that occur between the compounds and the proteins which underscore the potency of the ligands. Based on the results (Table 3.2), the differential total binding free energies ΔG_{bind} computed for 1,2,3-benzenetriol, α -D-glucopyranoside methyl, guanosine, phytol, sucrose, cinnamaldehyde and quercetin bound to 3QP1 were -21.59 kcal/mol, -17.48 kcal/mol, -18.34 kcal/mol, -27.12 kcal/mol, -23.40 kcal/mol, -14.43 kcal/mol and -35.97 kcal/mol respectively.

Table 3.2: Binding free energy estimates for the simulated complexes.

Compound name	ΔE_{vdw}	ΔE_{ele}	ΔG_{gas}	ΔG_{sol}	ΔG_{bind}
α -D-glucopyranoside, methyl	-17.84 $\pm$ 0.19	-26.65 $\pm$ 0.44	-44.49 $\pm$ 0.45	27.00 $\pm$ 0.32	-17.48 $\pm$ 0.19
1,2,3-Benzenetriol	-9.11 $\pm$ 0.28	-33.20 $\pm$ 1.00	-42.32 $\pm$ 1.01	20.73 $\pm$ 0.51	-21.59 $\pm$ 0.53
Guanosine	-22.52 $\pm$ 0.51	-22.54 $\pm$ 1.13	-45.06 $\pm$ 1.39	26.71 $\pm$ 0.96	-18.34 $\pm$ 0.52
Phytol	-31.90 $\pm$ 0.23	-4.06 $\pm$ 0.35	-35.96 $\pm$ 0.47	8.84 $\pm$ 0.31	-27.12 $\pm$ 0.26
Sucrose	-21.64 $\pm$ 0.28	-33.05 $\pm$ 1.02	-54.69 $\pm$ 1.07	31.29 $\pm$ 0.70	-23.40 $\pm$ 0.45
Reference compounds					
Cinnamaldehyde	-20.76 $\pm$ 0.12	-7.61 $\pm$ 0.40	-28.37 $\pm$ 0.39	13.54 $\pm$ 0.23	-14.83 $\pm$ 0.21
Quercetin	-39.13 $\pm$ 0.19	-25.79 $\pm$ 0.42	-64.91 $\pm$ 0.41	28.95 $\pm$ 0.24	-35.97 $\pm$ 0.25

3.4. DISCUSSION

The advances in QSIs as therapeutics present good tactics and it becomes imperative to explore the binding of a signal antagonist to a receptor, to comprehend its inhibitory effects (Aliyu et al., 2016). In this study, the MD approach was employed to reveal the potential of the identified compounds to inhibit the QS system in *C. violaceum*. The application of computer-based molecular modelling techniques to detect QSIs as anti-pathogenic agents and assess their bioactivity mechanism which includes multiple conformations could assist in predicting the binding affinity of the explored phytochemicals (Deryabin et al., 2019).

The feasibility of the docking protocol was inspected by re-docking the native ligand (C10-HSL) to the active site of the CviR' protein. The compounds of 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one, linalool, neophytadiene, phytol and pyroglutamic acid demonstrated weaker binding affinities as per their docking scores (Table 3.1). Whereas sucrose, decanedioic acid, guanosine, α -D-glucopyranoside, methyl and 1,2,3-benzenetriol revealed good binding affinities to the CviR' protein. When compared to the current study, Alyousef et al. (2021) demonstrated that linalool had good binding affinities with the CviR' protein while in this study, weaker binding affinities were observed. Alyousef et al. (2021) used, the CviR' protein of *C. violaceum* (ATCC 12472), a similar receptor protein was used in this study, although the crystal structure of the CviR' protein is unspecified. This could be a possible reason for the varying binding affinities of the same compound, however possibly different crystal structures were used.

The following amino acid residues of Trp84 form a direct H-bond with the lactone carbonyl while the acyl group –NH forms an H-bond with Asp97 and the carbonyl oxygen forms H-bonds with Tyr80 and Ser155 in the CviR' protein (Bodede et al., 2018). In this study, the results showed that 1,2,3-benzenetriol bound to the same active site as the native ligand. This suggests that 1,2,3-benzenetriol could play a role as a competitive inhibitor for the QS signalling compound thus binding to the AHL-binding pockets of the CviR' protein of *C. violaceum* (Bodede et al., 2018). Furthermore, 1,2,3-benzenetriol was the only compound that showed π - π staking interaction with Tyr80 while cinnamaldehyde also formed π - π staking interaction with Tyr88 and quercetin showed π - π staking interaction with both Tyr80 and Tyr88. According to Gupta & Bajaj (2018), the π - π stacking interaction illustrates a good

binding connection of the ligand to the receptor protein. Moreover, the compounds of guanosine and α -D-glucopyranoside, methyl were both shown to form H-bonds with Met135 and Trp84, indicating that they can bind to a similar binding pocket. Molecular modelling suggests that ligands in possession of an AHL antagonistic potential could possess a five-membered lactone ring with an acyl group as a spacer and a hydrophobic tail which facilitates binding of the ligands with the active site by H-bonding and hydrophobic interactions (Singh et al., 2015). Overall, the following compounds of sucrose > guanosine > α -D-glucopyranoside, methyl and 1,2,3-benzenetriol possess lactone rings with functional groups of COOH and OH which covalently formed hydrogen bonds with the amino acid residues of the CviR' protein and were able to reveal good docking scores. This may be attributed to the compounds' ability to fit in the AHL receptor's pocket due to their structural similarity.

As mentioned above, the best suitable QSI molecules depend on the compound's structure and the capability of non-competitive or competitive inhibition by targeting the active site (Paul, Guzman & Padilla, 2017). Several studies (Geske et al. (2007); Galloway et al. (2011); Singh et al. (2015)) suggest the structure and length of the hydrophobic functionalities can affect the potency and the intrinsic activity of the given ligand. In this study, the binding affinities of the best-docked ligands ranged between -23.61 to 39.29 kcal/mol. Aliyu et al. (2016) used a similar *in silico* molecular docking approach to our study, by showing four blumeoidolides with varying binding affinities (-20.00 to 44.50 kcal/mol) to CviR' protein. Quecan et al. (2019) displayed that quercetin aglycone and C10-HSL had a common residue of Ser155. Similarly in this study, the reference compound of quercetin was also shown to have the common residue of Ser155 as C10-HSL. Molecular docking revealed the potential binding of the identified compounds to QS-related receptor protein of *C. violaceum* which was indicative of a potential antagonistic effect, thereby disrupting the QS systems of the pathogen. Thus, to further comprehend the best-docked complexes' stable nature and their chemical properties several simulated trajectories were evaluated, this was performed because a protein is considerably affected by its stability and flexibility (Teilum, Olsen & Kragelund, 2011). In this study, the stability of the protein-ligand complex was measured through the root mean square deviation (RMSD). Ramírez & Caballero (2018), established RMSD classification for docking solutions with $\text{RMSD} \leq 2.00 \text{ \AA}$ considered good solutions, between 2.00 and 3.00 $\text{\AA}$ are acceptable solutions and

when $\text{RMSD} \geq 3.00 \text{ \AA}$ is considered a bad solution. Based on the results, the systems showed RMSD values between $1.64 \text{ \AA}$ and $3.00 \text{ \AA}$ which all showed good and acceptable solutions. All systems stabilized except for guanosine ($3.00 \text{ \AA}$) which did not fully stabilize for most of the simulation until the later stage. Also, α -D-glucopyranoside ($1.61 \text{ \AA}$) showed to be the most stable even more stable than both reference compounds. Shimu et al. (2022) indicated a similar trend with some of the docked complexes stabilizing after a certain period until the final stage. This stability confirms the reliability of our simulated systems. These systems were further evaluated for their flexibility by measuring the root-mean-square-fluctuation (RMSF). The RMSF values showed that α -D-glucopyranoside, methyl was the only ligand that was less flexible compared to the Apo. Generally, protein flexibility is necessary for adaptation to environmental changes, ligand binding, and chemical modifications. As such any perturbation that changes the flexibility of a protein may interfere with its function (Teilum, Olsen & Kragelund, 2011).

The binding energies are crucial in the binding and stability of small molecules to their target and therefore reveals the binding affinities, spontaneity, permeation, and reaction kinetics of the complex systems (Aldeghi, Gapsys & De Groot, 2018). The MM/GBSA method is a very popular technique for predicting binding energies and is known to be more accurate than most scoring functions in molecular docking and computationally less demanding than the alchemical free energy method (Genheden & Ryde, 2015). A lower negative binding free energy of a ligand indicates that the ligand has better binding abilities (Shimu et al., 2022). The compounds of phytol (-27.12 kcal/mol) and 1,2,3-benzenetriol (-21.59 kcal/mol) have demonstrated to have better binding abilities than the other compounds. Generally, the *in-silico* method for the biomonitor strain of *C. violaceum* presented the binding affinities of the CviR'-binding pocket and molecular modelling mode of action of phytochemical compounds. This molecular modelling approach showed that the investigated compounds could be used to target other bacterial QS receptor proteins in the attenuation of MDR pathogens.

3.5. CONCLUSION

In-silico modelling provide insight into identifying and validating novel compounds that are capable of inactivating or activating regulator proteins while decreasing cost and time. The advancement in *in-silico* methods provided a distinctive prospect for understanding the structure-activity relationship (SAR) of compounds with the respective receptor protein of the biomonitor strain. This shows the ability of novel compounds as promising QS inhibitors. These findings suggest that the investigated compounds (sucrose, guanosine, α -D-glucopyranoside, methyl, phytol and 1,2,3-benzenetriol) could potentially interfere with the QS protein of *C. violaceum* and expand the purposes of QSIs, which can modulate bacterial pathogenicity, virulence and resistance, without selective pressure, particularly in Gram-negative bacteria (GNB). The findings thus prompt the focus on targeting the QS-associated virulence factors and biofilm formation. Furthermore, searching for plant-derived compounds such as QSIs promotes the reduction of the upsurge in antimicrobial resistance.

In the next chapter, active plant extracts and respective phytochemical compounds are assessed for their potential to reduce the biofilm formation of selected MDR bacteria and in the latter chapter validate the potential of these compounds in QS-associated virulence of *Pseudomonas*, as a model GNB (MDR) pathogen.

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CHAPTER FOUR

***IN VITRO* AND *IN SITU* REDUCTION OF BIOFILM IN SELECTED MULTI-DRUG RESISTANT BACTERIA BY ACTIVE PLANT EXTRACTS AND PHYTOCHEMICALS**

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4.1. INTRODUCTION

Bacterial diseases are primarily associated with the ability of bacteria to inhabit and spread across their hosts through diverse motility forms, activating various virulence factors, and developing a well-structured biofilm; leading to cell and tissue damage, moreover evasion of the host's immune system (Carette, Nachtergaeel & Rasamiravaka, 2020). Biofilms are a diverse three-dimensional consortium of microbes that attaches to surfaces and have a sheath layer of exopolymetric substance (EPS) (Verderosa, Totsika & Fairfull-Smith, 2019; Zea et al., 2020). It is estimated that nearly 80% of the globe's microbial biomass is in a form of biofilms, whereas in nature the planktonic cells are deemed as the main mode of existence for pathogens (Miquel et al., 2016). Moreover, biofilms reveal phenotypes that are unique from their incoherent free-floating cells and have a greater ability to settle on surfaces and withstand exogenous stress (Miquel et al., 2016).

Numerous pathogenic bacteria such as *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* use quorum sensing (QS) approaches to trigger resistance genes and develop biofilm and their associated virulence factors (Doi et al., 2017). The multifaceted mechanisms of microorganisms have led to the acquired resistance to antibiotics, including reduced cell membrane permeability, modification of the molecular target, increased expression of efflux pump, and degradation of the antibiotics are all important factors that reduce drug efficacy on free planktonic cells (Borges et al., 2016). More so, alteration of the drug also contributes to the induction of cell biofilm-specific phenotypes, known as persister cells (Borges et al., 2016).

The formation of a biofilm involves factors such as EPS, cell motility, cell appendages and surface proteins controlled by second messengers of cyclic adenosine monophosphate (cAMP) and cyclic dimeric guanosine monophosphate (c-di-GMP). These factors perform a vital function in a communication network with the environment (Toyofuku et al., 2016). Microbial adhesion, following the assembly and buildup of an extracellular matrix comprised of several polymeric substances such as humic substances, extracellular DNA, proteins and polysaccharides make up the stages in the formation of biofilms (Azeredo et al., 2017).

Antibiofilm agents hinder bacterial virulence with no effect on the cell's viability (Tamfu et al., 2020). Guzzo et al. (2020) documented that antibiofilm agents derived from marine organisms, bacteria, plants, and fungi demonstrate efficacy in preventing the formation of EPS, cell attachment and diminishing virulence factors by disrupting the QS system.

Therefore, there is an increasing need to research for agents or compounds with novel antivirulence/antipathogenic properties, particularly by blocking bacterial QS systems thereby regulating virulence factors including biofilm formation (Álvarez-Martínez, Barraón-Catalán & Micol, 2020). Antibiofilm agents with the potential to prevent early biofilm formation provide the possibility of reducing microbial surface settlement (Bhandari et al., 2020). An incorporated part of the drug innovation and advancement process involves choosing the precise therapeutic target through high throughput screening (HTS) of hit-compounds for their absorption, distribution, metabolism and excretion (ADME) properties (Matias et al., 2017).

In this chapter, identified compounds from the bioactive extracts were evaluated for their antibacterial activities against MDR bacteria. Thereafter, the active extracts and compounds were assessed for their potential to prevent cell adhesion and preformed biofilms of MDR bacteria. Validation of preformed biofilm inhibition was examined using the *in-situ* visualization (scanning electron microscope). Furthermore, the safe use of plant extracts and compounds was evaluated using the *in-vitro* cytotoxicity assays and drug-likeness properties.

4.2. MATERIALS AND METHODS

4.2.1. Phytochemical extracts and compounds

Active extracts of *M. comosus* (aqueous, methanol and dichloromethane), *P. sidoides* (aqueous) and *V. karroo* (aqueous and methanol) with their antibacterial activities reported in chapter 2 were investigated further for their antibiofilm activity against selected MDR bacteria. These active extracts and compounds of guanosine and phytol which showed significant bioactivity were screened further for their cytotoxicity activities. The dried extracts and compounds were re-dissolved in 1% dimethyl sulfoxide (DMSO) at a final concentration of 100 mg/mL and 10 mg/mL,

respectively by sonication (Starsonic 90). Thereafter, filtered through a sterile membrane filter unit (0.45 μm , Merck Millipore) for better solubility and to remove any residuals. The stock solutions of the plant extracts and compounds were reconstituted to varying concentrations dilutions of 6.25–0.02mg/mL and 0.25 mg/mL– 0.0019 mg/mL for extracts and compounds, respectively supplemented with a complete medium. The following chemical compounds of 1,2,3-benzenetriol (Lot no: BCCG0904), guanosine (Lot no: BCCB9660) and phytol (Lot no: 0001452396) are some of the identified phytochemicals from medicinal plants of *M. comosus*, *P. sidoides* and *V. karroo*. These compounds were purchased from Sigma-Aldrich (Johannesburg, South Africa).

4.2.2. Cell culture and maintenance

African green monkey kidney (Vero ATCC CCL-81), epithelial and no malignant cells were cultivated in Eagle's Minimal Essential Medium supplemented with 10% FBS (Fetal Bovine Serum), 100 U/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin. Cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C. Before the experiments, the exhausted medium was discarded and cells were washed with phosphate-buffered saline (PBS; pH 7.2). Subsequently, Trypsin EDTA solution was added to the flask, to detach the cells from the surfaces. Cells were observed under a light microscope (Televal 31, Zeiss, Germany) at a 10x magnification to ensure that the cell layer is dispersed.

4.2.3. Determination of minimum inhibitory concentration (MIC) of selected chemical compounds against multidrug-resistant (MDR) bacteria

The antibacterial activity method was followed as described in section 2.2.3. Pathogens of *K. pneumoniae*, *E. coli*, *P. aeruginosa* *S. pyogenes*, and *S. aureus* were evaluated for minimum inhibitory concentration using three compounds, tested at a stock concentration of 1 mg/mL.

4.2.4. Inhibitory effects of plant extracts on cell attachment and biofilm development

The antibiofilm assay for both cell attachment and biofilm growth inhibition were assessed for selected plant extracts and compounds following the procedure described by Sandasi et al. (2011) with minor modifications. Succinctly, six extracts of *M. comosus* (aqueous, methanol and dichloromethane), *P. sidoides* (aqueous) and *V. karroo* (aqueous and methanol) and two compounds which showed significant MIC values (≤ 1 mg/mL; extracts and ≤ 0.064 mg/mL; compounds) were tested against *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus* and *S. pyogenes* for both cell attachment and biofilm development inhibition.

In the cell attachment inhibition assay, 100 μ L of standardized bacterial suspension ($OD_{600nm} = 0.1$), 100 μ L of MH broth and 100 μ L of extract were added to the wells. The positive control (ciprofloxacin, 0.001 mg/mL) and negative control (1% DMSO) were also added into the wells. The blank wells were added with 200 μ L of sterile MH broth and thereafter, incubated at 37 °C for 24 h. For biofilm development bioassays, 100 μ L of standardized bacterial suspension and 100 μ L of MH broth was added to the wells and incubated at 37 °C for 8 h. After incubation, 100 μ L of extracts and controls were transferred into respective wells and incubated further for 24 h. Biofilm biomass was assessed using the modified crystal violet (CV) assay. The 96-well plates containing formed biofilm were washed with sterile distilled water to remove planktonic cells and media. The plates were then oven-dried at 60 °C for 45 min. Following drying, 1% CV solution (Sigma-Aldrich, Johannesburg, South Africa) was used to stain the remaining biofilm for 15 min in the dark. The wells were then washed with sterile distilled water to remove any unabsorbed stain. A semiquantitative assessment of biofilm formation was performed by adding 125 μ L of 95% ethanol to destain the wells. In total, 100 μ L of the destaining solution was transferred to a new plate and the absorbance (OD_{585nm}) was determined using a multimode microplate reader (SpectraMax® paradigm).

The percentage of inhibition was determined using equation 2. The following criterion for interpretation of results was used; whereby values between 0 and 100% were interpreted as an inhibitory activity, then further, breaking it down as follows: values between 0 - 39% (weak activity), 40 - 69% (moderate activity), 70 -100%

(good activity) while negative values reflect the enhancement of growth instead of biofilm inhibition.

4.2.5. *In-situ* visualization of biofilm using a scanning electron microscope (SEM)

A scanning electron microscope (SEM) was used to investigate the structural modifications of biofilms after treatment with extracts and compounds. Biofilms were grown on coverslips, placed in 12-well polystyrene plates and incubated at 37 °C for 8 h. After 8h incubation, the preformed biofilm was supplemented with plant extracts, compounds and the positive control, (ciprofloxacin) then incubated further for 24 h at 37 °C. Following incubation, plates were rinsed three times with phosphate-buffered saline (PBS; pH 7.2) for 10 minutes. The biofilm grown on coverslips was fixed using 2.5% glutaraldehyde for 1-2 h at room temperature. Thereafter, coverslips were dehydrated by using graded series of ethanol (30, 50, 75, 90 and 100%) for 10 min each rinsing at room temperature. Thereafter, coverslips containing biofilms were allowed to dry overnight, then samples were mounted onto aluminium studs and coated with carbon and visualised using a crossbeam 540 scanning electron microscope (Carla Zeiss, Germany). The SEM images were recorded at 2000x and 5000x magnification

4.2.6. Topographical characterization of bacterial biofilms using atomic force microscopy (AFM)

The effect of the best three compounds (guanosine, phytol and quercetin) were monitored on *P. aeruginosa*, *S. aureus* and *S. pyogenes* using atomic force microscopy (AFM) as described by Santana et al. (2012), with minor modifications. The overnight bacterial strains were grown in LB media, centrifuged (2000 × *g*, room temperature, 15 min) and rinsed thrice using phosphate buffer solution (5 mM, pH 6.5). Tubes comprising the same buffer were resuspended with approximately 10⁸ colony-forming units (CFU/mL). The compounds were diluted using their respective MIC values and 100 µL of compounds were transferred to 3 mL of the cell suspensions. The samples were incubated for 4 hours at 37 °C. Untreated cell suspensions were used as controls. After incubation, 1 mL from each of the treated

samples was collected and centrifuged ($6000 \times g$) for 15 min at room temperature. A smear of cells was prepared on a glass slide. The slides were air-dried and visualized using the atomic force microscope. Samples were viewed in a contact imaging mode using a Veeco atomic force microscope (Dimension icon with Scan Asyst) and silicon tip on nitride lever (cantilever $0.55\text{--}0.75\text{ }\mu\text{m}$). A nominal constant of 32 Nm^{-1} and resonance frequency of $\approx 300\text{ kHz}$ was used with a scan rate of 0.100 Hz and scan size of $5.00\text{ }\mu\text{m}$. Nanoscope analysis Scan Asyst software (Nanoscope version 8.15) was used for the imaging analysis.

4.2.7. Evaluation of cytotoxic effects of selected extracts and compounds on mammalian cells

4.2.7.1. *Water-soluble tetrazolium salt (WST-8) and lactate dehydrogenase (LDH)*

Water-soluble tetrazolium salt (WST-8) was used to evaluate the metabolic activity of cells as an indicator of cell viability using the cell counting kit -8 (CCK-8; Dojindo Molecular 150 Technologies, Italy). WST-8 (colourless) is reduced by dehydrogenases present in cells to form an orange-coloured formazan, which is soluble in the tissue culture medium (Figure 4.1). The quantity of the formazan dye produced by the dehydrogenases in cells is directly proportional to the number of viable cells (Kamiloglu et al., 2020). The cytotoxicity activity was determined using the lactate dehydrogenase enzyme (LDH Assay kit-WST, Dojindo Molecular Technologies, Italy). LDH is a firm cytoplasmic enzyme released into the cell culture medium because of the loss of membrane integrity (Kamiloglu et al., 2020). LDH breaks down the lactate to pyruvate thus decreasing NAD to Nicotinamide adenine dinucleotide hydrogen (NADH). NADH reduces the WST in the presence of an electron mediator to form an orange formazan dye (Figure 4.2). The amount of LDH activity in the culture medium is directly proportional to the sum of damaged cells

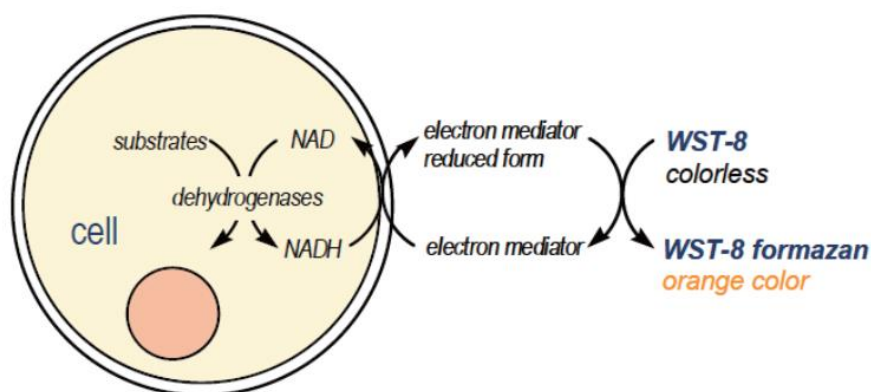


Figure 4.1: Image showing water-soluble tetrazolium salt (WST-8) (colourless) being reduced by dehydrogenases inside the cells and producing an orange-coloured product (formazan), which is soluble in the tissue culture medium

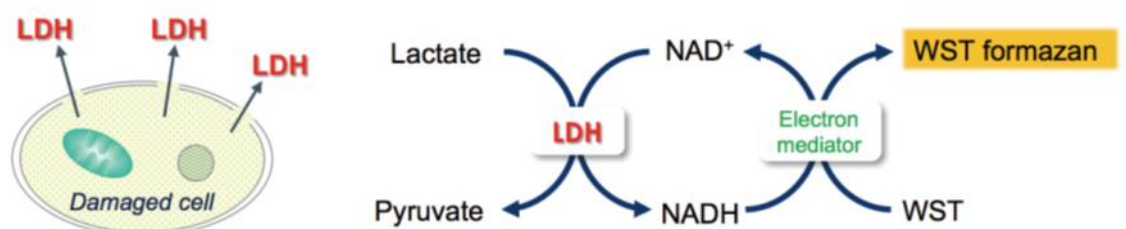


Figure 4.2: Lactate dehydrogenase that is released from the damaged cell oxidizes lactate to generate nicotinamide adenine dinucleotide hydrogen, which then reacts with WST to form the orange formazan

4.2.7.2. In-vitro cytotoxicity assays

Bioactive plant extracts and compounds were evaluated for their cytotoxic effects on epithelial African green monkey kidney cells (Vero ATCC CCL-81) following the method as described by Bonvicini et al. (2014), with slight modifications. CCK-8 and LDH release were assessed on mammalian cells after 48 h of treatment. Succinctly, 24 h before experiments, trypan blue was used for cell counting using the Bürker chamber to determine the number of cells to be seeded on the 96-well microplates. An aliquot of 100 μ L cell suspension was seeded into 96-well microplates at 10^4 cells/well and incubated in a 5% CO_2 at 37°C for 24 h. After incubation, the exhausted medium was replaced with the renewal of a complete medium supplemented with varying concentrations of extracts (6.25 – 0.02 mg/mL), compounds (0.25 – 0.0019 mg/mL) and doxorubicin (0.025 – 0.00019 mg/mL) then incubated further for 48h. All experiments included wells with untreated Vero cells

(positive control) background medium of the extracts and 1% DMSO (0.1 – 0.0008%). These varying concentrations of DMSO relative to the redissolved plant extracts were added to verify that the DMSO does not have any influence on the Vero cells. After 48 h incubation, cellular morphology evaluation of the micro-wells containing cells with treatment was visualised at a 10X magnification and captured using the Hamamatsu corporation image software (Olympus i73 mic). Subsequently, 10 µL of lysis buffer was transferred to several wells of Vero cells representing the positive control in the LDH and incubated for 10 min at 37°C. All supernatants were removed from the 96-well plate and stored for LDH measurements. Hundred (100) µL of fresh medium containing 10 µL of CCK-8 solution were added to the cell monolayer and incubated for 2 h. The optical density (OD) values at 450 nm were measured after 2 h of incubation and data were expressed as percentage values of cell viability relative to the untreated controls using equation 1.

In parallel, the collected cell-free supernatants were assayed for LDH released through damaged plasma membranes by adding a volume of the reconstituted working solution. After 30 min of incubation at room temperature in the dark, the stop solution was added and absorbance was measured at 490 nm. Data were expressed as percentage values relative to both the 100% lysis controls, included in the test, and untreated controls using equation 2. Plates were read at 490 nm using a Multiskan Ascent Microplate Reader (Thermo Fischer, Italy). The selectivity index (SI) was determined using the minimum inhibitory concentration (MIC) values of the extracts and compounds against the five tested pathogens and the cytotoxicity concentration (CC₅₀) values of the extracts against the Vero cells using equation 3.

$$\text{Cell viability (\%)} = \frac{\text{OD(450nm) untreated cells}}{\text{OD (450 nm) treated cells}} \times 100 \quad (1)$$

$$\text{Cytotoxicity (\%)} = \frac{(A-C)}{(B-C)} \times 100 \quad (2)$$

$$\text{SI} = \frac{\text{CC}_{50}}{\text{MIC}} \quad (3)$$

Where: A indicates treated cells (test samples), B: high control (lysed Vero cells) and C: low control (untreated Vero cells). CC: cytotoxic concentration and MIC: minimum inhibitory concentration.

4.2.8. *In-silico* screening of drug-likeness properties of selected chemical compounds

The evaluation of the pharmacokinetic and physicochemical properties of hit compounds was performed using the SwissADME web server (<http://www.swissadme.ch/index.php>) (Diana, Michielin & Zoete, 2017). The compound's smiles were obtained from the PubChem database and inserted in the webserver for analysis to generate the predicted parameters. Determination of water solubility, medicinal chemistry and lipophilicity of the compounds was generated. The bioavailability scores and drug-likeness properties (Lipinski's, Ghose's, Veber's, Egan's and Muegge's rules) of the compounds were calculated.

4.2.9. Statistical analysis

All the experiments were done in triplicate of at least two independent experiments. The results are presented as mean $\pm$ standard by Microsoft Excel. The values of extracts, compounds and positive controls were adjusted for comparisons using the pairwise comparisons of the one-way ANOVA - Turkey's method and used the t-student test for comparison among two groups. The 50% cytotoxicity concentration (CC₅₀) values were determined using the GraphPad Prism Software, version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). P values < 0.05 were considered significant. The following criteria for antibacterial activity of compounds was used, MIC < 0.01 mg/mL (noteworthy activity), 0.01 mg/mL < MIC $\leq$ 0.1 mg/mL (moderate activity), and MIC > 0.1 mg/mL (insignificant) (Kuate, 2010).

4.3. RESULTS

4.3.1. Antibacterial activity of selected chemical compounds against MDR bacteria

The three phytochemical compounds were evaluated for their antibacterial activity on selected MDR pathogens. The MICs for the three tested compounds of 1,2,3-benzenetriol, guanosine and phytol revealed MIC values ranging from 0.25 – 0.004 mg/mL against the five selected MDR bacteria (Table 4.1). The Gram-positive bacteria (GPB) of *S. pyogenes* and *S. aureus* were the most susceptible to the treatment. For *S. pyogenes*, a significant MIC value of 0.016 mg/mL was observed for both guanosine and phytol while 1,2,3-benzenetriol, showed a MIC value of 0.031 mg/mL. All three compounds of 1,2,3-benzenetriol, guanosine and phytol, revealed an MIC value of 0.031 mg/mL against *S. aureus*. The Gram-negative bacteria (GNB) (*E. coli*, *K. pneumoniae* and *P. aeruginosa*) were less susceptible to the treatment, all compounds showed MIC values of 0.250 mg/mL for both *E. coli* and *K. pneumoniae*. A noteworthy MIC value of 0.031 mg/mL and 0.063 mg/mL was shown by guanosine and phytol, respectively while 1,2,3-benzenetriol showed MIC of 0.125 mg/mL against *P. aeruginosa*. The best compounds that showed significant MIC values were guanosine, followed by phytol and 1,2,3-benzenetriol against all five tested bacteria.

Table 4.1: Minimum inhibitory concentrations of compounds against multidrug-resistant bacteria

Compounds	Antibacterial activities MIC (mg/mL)				
	<i>E. coli</i> ATCC 10536	<i>K. pneumoniae</i> ATCC 33495	<i>P. aeruginosa</i> ATCC 9721	<i>S. aureus</i> ATCC 25923	<i>S. pyogenes</i> ATCC 19615
1,2,3-Benzenetriol	0.250	0.250	0.125	0.031	0.031
Guanosine	0.250	0.250	0.031	0.031	0.016
Phytol	0.250	0.250	0.063	0.031	0.016
Quercetin	0.250	0.250	0.008	0.004	0.004

4.3.2. Cell attachment inhibition of plant extracts and compounds

Cell attachment (anti-adhesion) inhibition activity was assessed for the six extracts and seven compounds at sub-MIC concentrations against the five tested bacteria (Table 4.2). The tested extracts were *Melanthus comosus* (aqueous, methanol and dichloromethane), *Pelargonium sidoides* (aqueous) and *Vachellia karroo* (aqueous and methanol) while the tested compounds were 1,2,3-benzenetriol, guanosine and phytol.

Based on the results, a 4.33 – 66.34% anti-adhesion inhibition was observed with the extracts against the five bacteria (Table 4.2). Moderate anti-adhesion inhibition was observed with the following extracts of *P. sidoides* (aqueous) and *M. comosus* (methanol) with percentage inhibition of 66.34%, and 50.28%, respectively. While the other four extracts of *M. comosus* (aqueous and dichloromethane), *V. karroo* (aqueous and methanol) showed weak biofilm inhibition between 0.64 – 49.53% against *S. pyogenes*. All three tested compounds showed good anti-adhesion inhibition against *S. pyogenes* (Table 4.2). Guanosine and phytol exhibited the best cell attachment inhibition with 76.89% and 76.84%, respectively while 1,2,3-benzenetriol showed a 51.52% inhibition against *S. pyogenes*. The positive control, ciprofloxacin also exhibited good inhibitory activity of 72.53% while quercetin showed 74.65% inhibitory activity against *S. pyogenes*. All six extracts exhibited $\pm 40\%$ weak inhibitory effect against *S. aureus* while the three tested compounds showed good to moderate inhibitory activity with 76.48, 72.61 and 50.10% for guanosine, phytol and 1,2,3-benzenetriol, respectively against *S. aureus*. Ciprofloxacin and quercetin exhibited moderate inhibitory activity of 61.34% and 61.65%, respectively. Compounds of guanosine and phytol performed even better ($p < 0.05$) than the positive controls against *S. aureus*.

Table 4.2: Anti-adhesion inhibition of plant extracts and compounds against multi-drug resistant bacteria

Plant species	Cell attachment percentage inhibition (%)				
	<i>E. coli</i> ATCC 10536	<i>K. pneumoniae</i> ATCC 33495	<i>P. aeruginosa</i> ATCC 9721	<i>S. aureus</i> ATCC 25923	<i>S. pyogenes</i> ATCC 19615
Aqueous extracts					
<i>M. comosus</i>	-	23.42±0.05 ^{a, b}	-	31.70±0.06 ^{a, b}	46.61±0.10 ^{b, c}
<i>P. sidoides</i>	-	9.17±0.05 ^a	-	39.60±0.06 ^b	66.34±0.22 ^c
<i>V. karroo</i>	-	-	-	32.09±0.06 ^{a, b}	40.38±0.10 ^b
Methanol extracts					
<i>M. comosus</i>	-	29.12±0.05 ^{a, b}	-	40.27±0.08 ^b	50.28±0.12 ^{b, c}
<i>V. karroo</i>	-	-	-	4.33±0.05 ^a	38.05±0.06 ^b
Dichloromethane extract					
<i>M. comosus</i>	-	0.64±0.05 ^a	-	35.34±0.06 ^b	49.53±0.10 ^{b, c}
Compounds					
1,2,3-benzenetriol	15.79±0.02 ^a	4.08±0.03 ^a	49.93±0.45 ^{b, c}	50.10±0.08 ^{b, c}	51.52±0.02 ^{b, c}
Guanosine	40.34±0.01 ^b	38.58±0.04 ^b	73.88±0.11 ^c	76.48±0.01 ^c	76.89±0.01 ^c
Phytol	31.26±0.04	41.57±0.02 ^b	70.20±0.00 ^c	72.61±0.03 ^c	76.84±0.01 ^c
Ciprofloxacin	53.48±0.18 ^{b, c}	59.30±0.15 ^{b, c}	52.88±0.18 ^{b, c}	61.34±0.20 ^c	72.53±0.22 ^c
Quercetin	33.02±0.05 ^{a, b}	29.92±0.03 ^{a, b}	55.14±0.03 ^{b, c}	61.65±0.03 ^c	74.65±0.03 ^c

Means are values of triplicate independent experiments ± SD. The contrast of percentage inhibition at 1 mg/mL and 0.250 mg/mL for every extract and compound, respectively against GPB (*S. pyogenes* and *S. aureus*) and GNB (*P. aeruginosa*, *E. coli* and *K. pneumoniae*). (-) No inhibition Various letters (a–c) show significant differences at p < 0.05.

The GNB (*E. coli*, *K. pneumoniae* and *P. aeruginosa*) presented some degree of resistance towards the extracts, exhibiting a weak activity of 0.64 – 29.12 % while for compounds there was weak and strong activity ranging between 6.25 – 73.88% (Table 4.2). Dichloromethane extract of *M. comosus* presented a less than 10% inhibition against *E. coli*, while the remaining extracts enhanced the growth (Table 4.2). The compounds of guanosine and phytol also exhibited weak activity of 40.34% and 31.26%, respectively against *E. coli*. With *K. pneumoniae*, four extracts displayed weak inhibition while two extracts were enhancing the growth of the bacterium. Similarly, this bacterium was also susceptible to the treatment of compounds, exhibiting weak inhibition. Guanosine and phytol exhibited a preformed biofilm reduction of 38.58 and 41.57%, respectively while quercetin showed a 29.92% inhibition, with a significant difference ($p < 0.05$) against *K. pneumoniae*. Furthermore, all extracts enhanced the growth of *P. aeruginosa* instead of anti-adhesion inhibition, while ciprofloxacin exhibited 52.88% inhibitory activity against *P. aeruginosa*. Strong inhibition of 73.88% and 70.20% was shown by guanosine and phytol, respectively against *P. aeruginosa*, both these compounds showed better activity than the positive control of ciprofloxacin (52.88%) and quercetin (55.14%) with significant difference ($p < 0.05$). Overall guanosine and phytol remained the best performing compounds for all five tested MDR bacteria.

4.3.3. Biofilm development inhibition of plant extracts and compounds

The six extracts were also evaluated for their effect to inhibit the mature biofilms of the five tested MDR bacteria. The extracts presented weak activity between 8.54 - 31.82% against the five tested bacteria (Table 4.3). The effects of plant extracts on biofilms were observed only in the Gram-positive bacteria whereas the Gram-negative bacteria did not exhibit inhibition but rather enhanced their growth. *M. comosus* (aqueous, methanol and dichloromethane) and *P. sidoides* (aqueous) extracts showed percentage inhibition of, 9.81%, 31.82%, 31.16% and 38.54%, respectively against *S. pyogenes*.

Methanolic and dichloromethane extracts of *M. comosus* were the only extracts that exhibited weak activity of 10.97% and 17.45% against *S. aureus*. While all extracts did not exhibit any inhibition for the growth of *E. coli*, *K. pneumoniae* and *P. aeruginosa*. The positive control, ciprofloxacin showed weak percentage

inhibition of 36.27%, 39.72% and 39.43% for *E. coli*, *K. pneumoniae* and *P. aeruginosa*, respectively.

The three tested compounds exhibited weak inhibitory activities against the five tested MDR pathogens (Table 4.3). All tested compounds exhibited a $\pm 30\%$ weak inhibitory effect on both GPB of *S. pyogenes* and *S. aureus*. Phytol presented a preformed biofilm inhibition of 39.71 and 35.33% for *S. pyogenes* and *S. aureus*, respectively. Followed by guanosine with 36.35% and 34.57% while 1,2,3-benzenetriol showed performed inhibition of 31.61% and 31.18% for *S. pyogenes* and *S. aureus*, respectively. Quercetin exhibited a percentage inhibition of 43.38% and 42.30% against *S. pyogenes* and *S. aureus*, respectively. The GNB also showed resistance to the treatment of compounds, with lower percentage inhibition. For *E. coli*, the weak activity of 5.47 – 21.73% was presented by the three tested compounds, whilst between 5.35 – 23.68% inhibitory effects were exhibited by the compounds against *K. pneumoniae*. Similarly, the tested compounds showed an inhibitory effect between 18.83 – 34.85% against *P. aeruginosa*. For preformed biofilm assessment, the tested bacteria exhibited some resistance towards the compounds.

Table 4.3: Biofilm development inhibition of plant extracts and compounds against multi-drug resistant bacteria

Biofilm development (%)					
Plant species	<i>E. coli</i> ATCC 10536	<i>K. pneumoniae</i> ATCC 33495	<i>P. aeruginosa</i> ATCC 9721	<i>S. aureus</i> ATCC 25923	<i>S. pyogenes</i> ATCC 19615
Aqueous extracts					
<i>M. comosus</i>	-		-	-	9.81±0.05 ^a
<i>P. sidoides</i>	-		-	-	38.54±0.05 ^b
<i>V. karroo</i>	-	-	-	-	-
Methanol extracts					
<i>M. comosus</i>	-		-	10.97±0.05 ^a	31.82±0.06 ^{a, b}
<i>V. karroo</i>	-	-	-	-	-
Dichloromethane extract					
<i>M. comosus</i>	-		-	17.45±0.05 ^a	31.16±0.06 ^{a, b}
Compounds					
1,2,3-benzenetriol	11.15±0.02 ^a	8.35±0.01 ^a	18.83±0.29 ^a	31.18±0.11 ^{a, b}	31.61±0.04 ^{a, b}
Guanosine	14.07±0.03 ^a	22.86±0.03 ^a	34.85±0.16 ^{a, b}	34.57±0.05 ^{a, b}	36.35±0.05 ^b
Phytol	21.73±0.03 ^a	24.84±0.04 ^a	32.79±0.01 ^{a, b}	35.33±0.02 ^b	39.71±0.05 ^b
Quercetin	15.35±0.00 ^a	23.68±0.02 ^a	44.35±0.05 ^c	42.30±0.03 ^c	43.38±0.04 ^c
Ciprofloxacin	39.72±0.06 ^b	36.27±0.06 ^b	39.43±0.06 ^b	40.90±0.08 ^b	49.02±0.10 ^c

Means are values of triplicate independent experiments ± SD. The contrast of percentage inhibition at 1 mg/mL and 0.250 mg/mL for every extract and compound, respectively against GPB (*S. aureus* and *S. pyogenes*) and GNB (*E. coli*, *K. pneumoniae* and *P. aeruginosa*). (-) No inhibition Various letters (a–c) show significant differences at $p < 0.05$.

4.3.4. Scanning electron microscopy analysis

The SEM analysis assisted with validation of the results obtained for biofilm development and capturing micrographs of biofilm layers formed by *E. coli* (Figure 4.3.1A), *K. pneumoniae* (Figure 4.3.1B), *P. aeruginosa* (Figure 4.3.1C), *S. aureus* (Figure 4.3.1D) and *S. pyogenes* (Figure 4.3.1E), without treatment (untreated control). The untreated controls (Figure 4.3.1A – 1E) were then exposed to treatment using the positive control of ciprofloxacin, which showed a disruption and reduction of the biofilm layers of the targeted bacteria (Figure 4.3.2A – 2E). Biofilm layers treated with plant extracts of *M. comosus* (methanol) (Figure 4.3.3A – 3E) and *P. sidoides* (aqueous) (Figure 4.3.4 – 4E), and compounds of guanosine (Figure 4.3.5A – 5E) and phytol (Figure 4.3.6A – 6E). The disruption of *S. aureus* and *S. pyogenes* biofilms were more observed from guanosine in Figure 4.3.5D and Figure 4.3.5E, respectively and phytol in Figure 4.3.6D and Figure 4.3.6E, respectively. Based on these overall micrographs, this corroborates with the weak inhibition obtained from the *in-vitro* biofilm development assay for plant extracts and compounds against the tested bacteria.

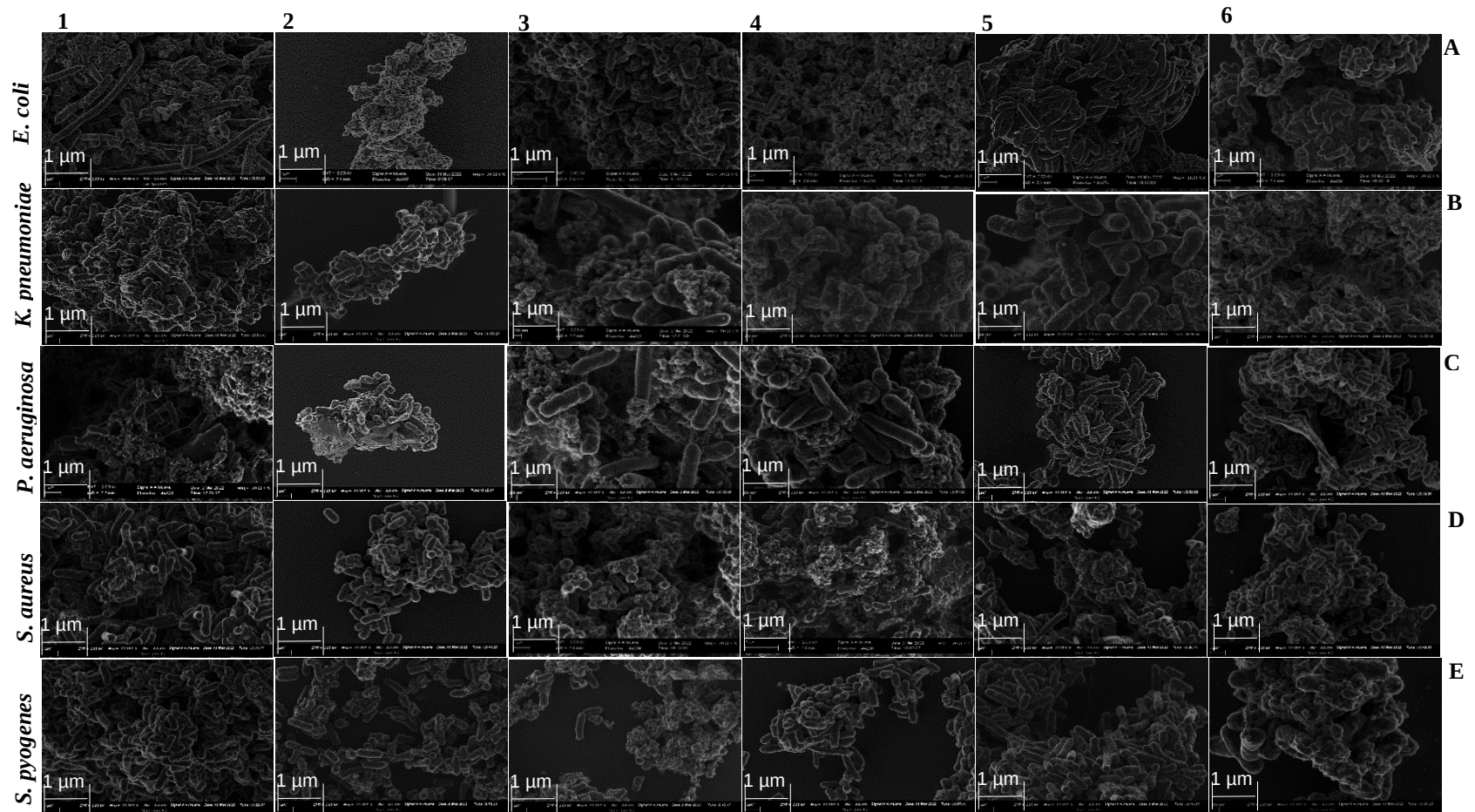


Figure 4.3: Micrograph images by scanning electron microscope following exposure to plant extracts (1 mg/mL and compounds (0.25 mg/mL) after 8 h biofilm development to validate their inhibitory effects on biofilms. Five bacteria were tested as shown in A: *E. coli*; B: *K. pneumoniae*; C: *P. aeruginosa*; D: *S. aureus* and E: *S. pyogenes*. 1: Untreated control; 2: Ciprofloxacin; the following plant extracts and compounds were tested: 3: *M. comosus* (methanol); 4: *P. sidoides* (aqueous); 5: Guanosine and 6: Phytol. SEM micrographs were recorded at 2000x and 5000x magnification

4.3.5. Atomic force microscopy analysis

The two and three-dimensional views of the surface topographies of studied *P. aeruginosa*, *S. aureus* and *S. pyogenes* biofilms are shown in Figure 4.4. Conspicuous differences were detected between the topographies of untreated and treated biofilms using the atomic force microscope (AFM). The AFM analysis revealed the surface roughness of biofilms formed by *P. aeruginosa*, *S. aureus* and *S. pyogenes*, mainly composed of unevenly distributed lumps (Figure 4.4 (A1, E1 and I1)). The biofilms showed a maximum height of 403.9nm for untreated *P. aeruginosa*, 332.4nm for untreated *S. aureus* and 445.3nm for untreated *S. pyogenes* as shown in the 2D images (Figure 4.4 (A1, E1 and I1)). An average roughness (Ra) of 28.7nm (*P. aeruginosa*), 80.6nm (*S. aureus*) and 96.2nm (*S. pyogenes*) is shown in the 3D images (Figure 4.4 (A2, E2 and I2)) were obtained using the nanoscope analysis (v 8.15) software.

Biofilms treated with guanosine and phytol at their respective MIC values revealed visible differences in height and surface roughness in comparison with the untreated biofilms. For biofilms treated with guanosine, maximum lump heights of 252.0nm, 243.0nm and 255.5nm were recorded for *P. aeruginosa*, *S. aureus* and *S. pyogenes* respectively (Figure 4.4 (B1, F1 and J1)). However, biofilms treated with phytol had a maximum height of 289.0 nm and 283.1 nm and 33.1nm for *P. aeruginosa*, *S. aureus* and *S. pyogenes* respectively. (Figure 4.4 (C1, G1, and K1)). The average roughness (Ra) for phytol-treated biofilms was 54.7nm for *P. aeruginosa*, 62.7nm for *S. aureus* and 2.68nm for *S. pyogenes*. Figure 4.4 (C2, G2 and K2) revealed a reduction in surface roughness when compared with the untreated biofilms.

Notably, biofilms treated with the positive control (quercetin) revealed the highest reduction in the surface roughness and height, with a maximum lump height at 204.3nm, 35.6nm and 11.6nm for *P. aeruginosa*, *S. aureus* and *S. pyogenes* respectively (Figure 4.4 (D2, H2 and L2)). Their average roughness was also remarkably reduced to 36.5nm, 4.29nm and 1.25nm.

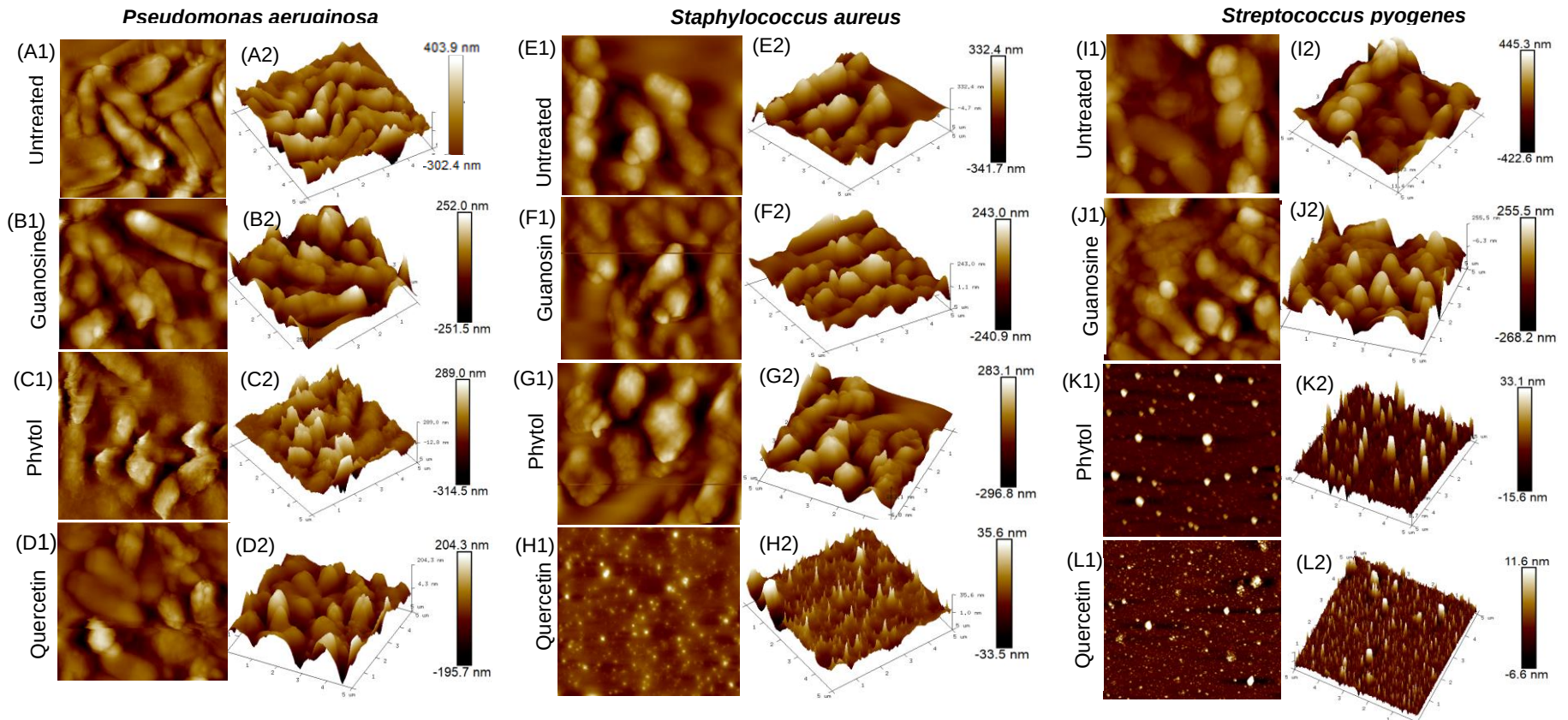


Figure 4.4: Atomic force microscope images showing two-dimensional (2D) and three-dimensional (3D) surface topography of untreated and treated biofilms of *P. aeruginosa*, *S. aureus* and *S. pyogenes* at a scan size of 5.00 μm (5000 nm). 2D images of untreated and treated biofilms are shown in A1–L1 while the 3D images are shown in A2–L2.

4.3.6. Cytotoxicity activities of bioactive extracts and compounds

The cytotoxicity test was carried out on Vero cells to determine the safety of extracts and compounds. Treatment with extracts and compounds exhibited a decrease in cell viability in a dose-dependent manner, whereby the extracts had a cytostatic effect on the cells at a higher concentration (Table 4.4). Since DMSO was tested to evaluate the effect, it will have on the cells and no cytotoxic effect was observed. Based on the results, the plant extracts showed a cytostatic effect on cells, *V. karroo* (aqueous and methanol) and *P. sidoides* (aqueous) were shown to have less effect on Vero cells as compared to *M. comosus* extracts (Table 4.4). The CC₅₀ values of *V. karroo* (aqueous and methanol) and *P. sidoides* (aqueous) extracts were 0.63, 0.79 and 0.59 mg/mL, respectively with significant difference ($p < 0.05$). Aqueous and methanolic extracts of *M. comosus* showed CC₅₀ values of 0.18 and 0.25 mg/mL, respectively (Table 4.4) with significant differences ($p < 0.05$). For the phytochemical compounds, both guanosine and phytol exhibited no cytotoxic effect on the Vero cells, approximately 100% ($p < 0.05$) of the cell's viability was maintained even at the highest tested concentration (0.25 mg/mL), as presented in Table 4.4.

The micrograph images display the morphological changes of the Vero cells after being treated with extracts and compounds (Figure 4.5). Figures 4.5A and 4.5B represent aqueous and methanolic extracts of *M. comosus* at 0.78 mg/mL and 0.39 mg/mL concentrations, respectively where it was shown that cells maintained their cellular morphology and presented cell viability of 31.56% and 50.05%, respectively. Whereas *V. karroo* (aqueous and methanol) extracts showed cell viability of 94.37% at 0.19 mg/mL and 95.95% at 0.39 mg/mL corresponding to Figures 4.5C and 4.5D, respectively. Aqueous extract of *P. sidoides* showed a percentage cell viability of 87.54% at 0.39 mg/mL (Figure 4.5E). Guanosine and phytol showed higher confluency of cells even at a lower concentration as presented in Figures 4.5F and 4.5G, respectively.

Table 4.4: Percentage (%) cell viability relative to the untreated Vero cells (ATCC CCL81) at different concentrations (mg/mL) of the extracts and cytotoxicity concentration at 50% (CC₅₀) values.

Concentrations (mg/mL)	Plant extracts					Concentrations (mg/mL)	Compounds		
	<i>M. comosus</i>		<i>P. sidoides</i>	<i>V. karroo</i>			Guanosine	Phytol	Doxorubicin
	AQ	ME	AQ	AQ	ME				
0.02	88.30±1.12	65.41±9.72	101.97±9.78	101.86±9.49	109.55±6.81	0.002	101.18±3.51	106.56±10.53	79,04±17,31
0.04	73.32±7.94	67.25±8.07	98.95±7.52	97.56±24.59	105.42±9.83	0.004	99.18±0.45	106.27±7.47	74,16±24,02
0.19	51.05±1.69	56.45±5.67	90.36±10.38	94.37±15.05	101.18±19.87	0.007	97.71±1.49	102.58±2.08	71,73±13,59
0.39	47.76±6.37	50.05±5.43	87.54±10.20	53.46±15.53	95.95±6.91	0.016	97.04±1.24	101.24±4.03	69,90±16,38
0.78	39.69±1.81	38.56±6.90	21.07±8.33	35.79±35.11	49.10±35.46	0.031	95.49±2.06	98.61±8.49	69,05±11,69
1.56	15.26±0.45	17.57±8.48	9.57±2.00	13.52±5.41	11.52±0.84	0.062	94.41±6.76	96.72±2.16	59,72±3,86
3.12	7.76±1.17	14.44±5.51	9.49±1.81	12.18±1.13	11.27±0.24	0.125	94.92±3.60	92.72±5.61	56,27±8,26
6.25	2.91±1.47	-3.22±6.98	0.69±0.50	-0.77±1.79	0.93±0.50	0.25	89.79±5.40	92.28±5.24	43,18±2,83
CC ₅₀	0.25	0.18	0.59	0.63	0.79				0.08

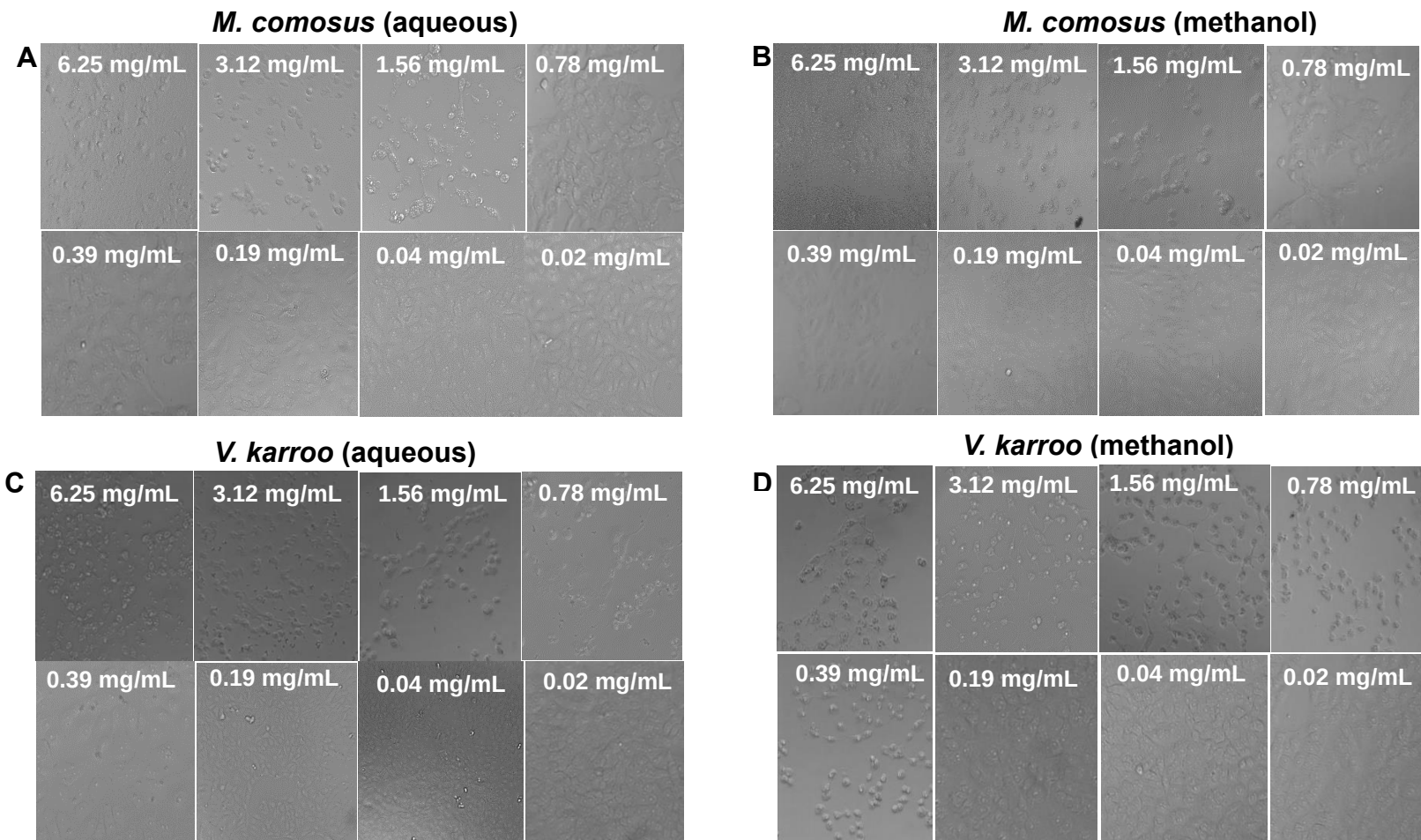
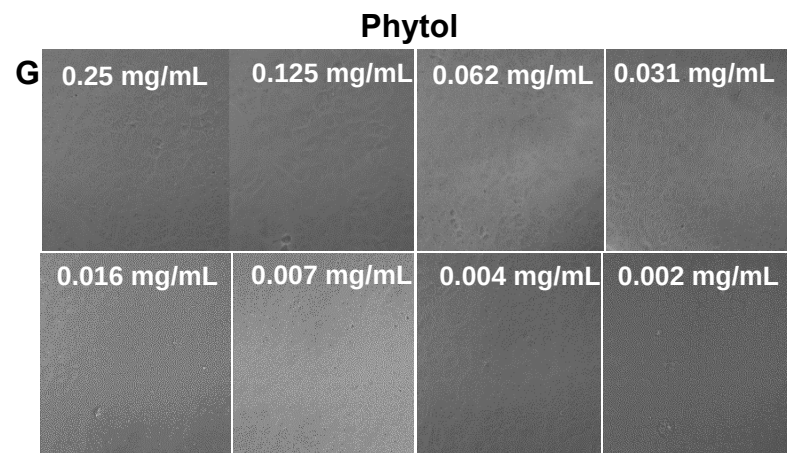
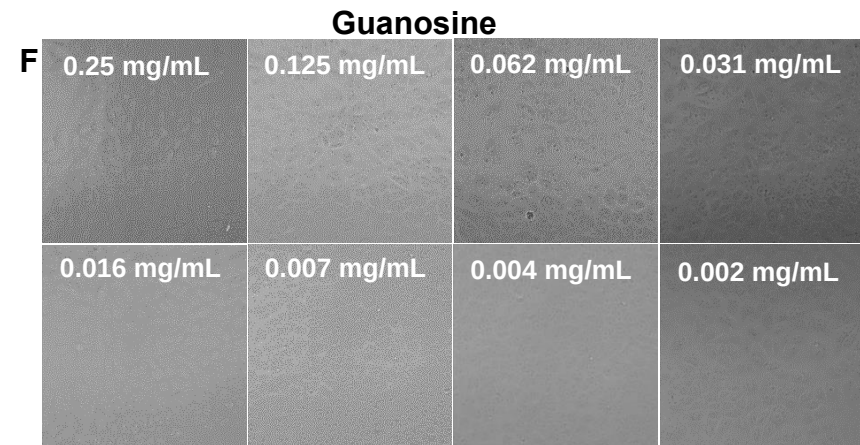
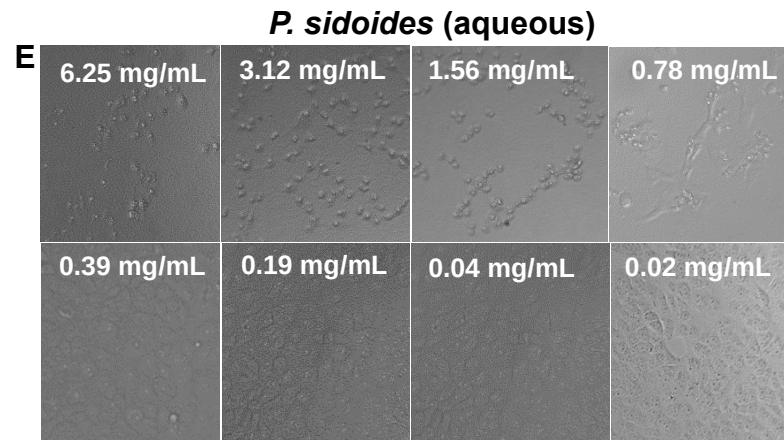


Figure 4.5: Micrograph images displaying Vero cells exposed to plant extracts and compounds for 48 h at varying concentrations of 6.25 – 0.02 mg/mL (extracts) and 0.25 -0.002 mg/mL (compounds). (A) *Melianthus comosus* (aqueous), (B) *Melianthus comosus* (methanol), (C) *Vachellia karroo* (aqueous) and (D) *Vachellia karroo* (methanol).



Vero cells

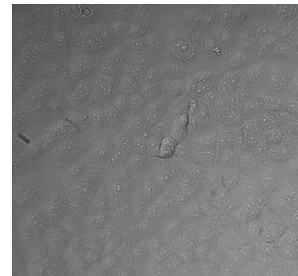


Figure 4.5 (continued): Micrograph images displaying Vero cells exposed to plant extracts and compounds for 48 h at varying concentrations of 6.25 – 0.02 mg/mL (extracts) and 0.25 -0.002 mg/mL (compounds). (E) *Pelargonium sidoides* (aqueous), (F) Guanosine and (G) Phytol.

A selectivity index value >1 indicates that the extract is more toxic to the pathogen than to mammalian cells. The higher the selectivity index value the less toxic the plant extract. The plant extracts were shown to have a less cytotoxic effect on the bacterial cells with the SI values ranging from 0.08 to 1.01 (Table 4.5). Cytotoxicity activity (LDH) of plant extracts and compounds is presented in Table 4.6. As the amount of released enzyme from cells is one of the major methods to assess cell death and only a negligible presence of lactate dehydrogenase enzyme was measured in our experimental conditions, results indicate that the extracts and compounds exerted a cytostatic effect on Vero metabolism rather than a cytotoxic effect.

Table 4.5: Selectivity index of selected plant extracts and compounds

Plant extracts	Selectivity index				
	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>P. aeruginosa</i>	<i>S. pyogenes</i>	<i>S. aureus</i>
Aqueous					
<i>M. comosus</i>	0.08	0.32	0.32	0.64	0.36
<i>P. sidoides</i>	0.09	0.09	0.09	0.76	0.19
<i>V. karroo</i>	0.10	0.40	0.20	0.81	0.40
Methanol					
<i>M. comosus</i>	0.06	0.23	0.23	0.95	0.95
<i>V. karroo</i>	0.13	0.13	0.25	1.01	0.25

Table 4.6: Lactate dehydrogenase activity on Vero cells (ATCC CCL81) at different concentrations of the extracts.

Concentration (mg/mL)	<i>M. comosus</i>		<i>P. sidoides</i>	<i>V. karroo</i>		Concentration (mg/mL)	Guanosine	Phytol	Doxorubicin
	AQ	ME	AQ	AQ	ME				
0.02	-8.39±2.81	1.41±02.5	-7.77±6.69	-5.03±1.80	6.32±4.91	0.002	6.31±3.00	2.08±1.17	-4.44±3,24
0.04	-7.38±3.40	-4.77±0.88	-6.89±3.16	-4.49±1.43	-2.50±2.13	0.004	1.91±0.72	-1.43±0.26	1,29±3,44
0.19	-8.16±3.60	-7.60±1.34	-14.73±2.67	-6.98±3.15	-6.29±3.93	0.007	-0.89±0.51	-1.61±0.45	8,90±1,90
0.39	-14.01±5.78	-12.98±2.30	-17.41±4.88	-9.48±0.38	-7.67±3.65	0.016	-3.39±1.75	-4.85±0.16	9,82±1,77
0.78	-14.97±3.17	-12.98±2.98	-3.22±2.52	-10.62±1.78	9.42±2.41	0.031	-0.51±0.36	-1.63±0.35	8,73±0,84
1.56	-8.99±5.24	-17.81±0.34	-2.89±2.52	11.59±3.74	24.46±3.05	0.062	-0.37±0.07	-1.69±0.18	16,50±5,43
3.12	-11.14±2.01	-7.97±6.15	-18.65±5.56	4.58±2.19	-11.72±0.53	0.125	-0.20±0.09	-0.75±0.27	22,53±3,93
6.25	-14.00±5.98	-4.89±7.47	-15.55±5.12	-11.30±4.99	-10.29±3.55	0.25	-2.59±0.34	-4.13±0.99	33,20±3,39

4.3.7. Drug-likeness predictions of the studied compounds

The *in-silico* ADME predictions of the three respective compounds of guanosine, phytol and quercetin (reference compound) were determined as presented in Table 4.7. Based on the analysis, both phytol and guanosine had low gastrointestinal (GI) absorption whereas quercetin has high GI absorption, guanosine and quercetin are P-glycoprotein substrates except for phytol and all three compounds cannot permeate the blood-brain barrier (BBB) with Log K_P values of -2.29, -7.05 and -9.37 cm/s for phytol, quercetin and guanosine, respectively (Table 4.7). Guanosine was predicted as a non-inhibitor of all cytochrome enzymes whereas phytol was an inhibitor for only CYP2C19 and quercetin was an inhibitor for CYP1A2, CYP2D6 and CYP3A4. Of the three compounds, guanosine appears to be the most soluble in water and phytol to be moderately soluble as shown by Log S (Esol and SILICOS-IT) in Table 4.7.

All three compounds had heavy metals of ≥ 20 , phytol had 13 rotational bonds and for both H-bond acceptors and donors was 1 with molar refractivity of 98.94 and TPSA of 20.23 Å². Conversely, guanosine had 2 rotational bonds, 7 and 5 H-bond acceptors and donors, respectively with molar refractivity of 65.50 and topological polar surface area (TPSA) of 159.51 Å². Additionally, quercetin obeyed all the rules, while guanosine and phytol disobeyed the Veber and Egan rules, all compounds had a bioavailability score of 0.55 F. Guanosine had zero alerts for both pan assay interference compounds (PAINS) and brek with synthetic accessibility of 3.85, in contrast, phytol had zero alert PAINS but 1 alert brek and synthetic accessibility of 4.30 then quercetin had 1 alert of catechol A for both PAINS and brek.

Table 4.7: Drug-likeness properties of the studied compounds

Compounds	Pharmacokinetics								
	GI absorption	BBB permeation	P-gp substrate	CYP1A2 Inhibitor	CYP2C19 Inhibitor	CYP2C9 Inhibitor	CYP2D6 Inhibitor	CYP3A4 Inhibitor	Log Kp (Skin permeation) (cm/s)
Phytol	Low	No	Yes	No	No	Yes	No	No	-2.29
Guanosine	Low	No	No	No	No	No	No	No	-9.37
Quercetin	High	No	No	Yes	No	No	Yes	Yes	-7.05
	Water solubility								
	Log S (Esol)	Solubility (mg/mL)	class	Log S (Ali)	Solubility (mg/mL)	Class	Log S (Silicos-it)	Solubility (mg/mL)	class
Phytol	-5.98	3.10e-04	MS	-8.47	9.94e-07	PS	-5.51	9.06e-04	MS
Guanosine	-0.61	7.01e+01	VS	-0.94	3.25e+0.1	VS	0.51	9.10e+0.2	S
Quercetin	-3.53	7.51e-02	S	-3.60	6.41e-02	S	-3.24	1.73e-01	S

VS: very soluble; MS: Moderately soluble; PS: poorly soluble; S: soluble

Table 4.7: Continued.

Physicochemical properties						
	No. heavy atoms	No. rotatable bonds	No. H-bond acceptors	No. H-bond donors	Molar Refractivity	TPSA (Å²)
Phytol	21	13	1	1	98.94	20.23
Guanosine	20	2	7	5	65.50	159.51
Quercetin	22	1	7	5	78.03	131.36
Drug-likeness						
	Lipinski	Ghose	Veber	Egan	Muegge	Bioavailability score
Phytol	Yes;1 violation: MLOGP>4.15	No; 1 violation: WLOGP>5.6	No; 1 violation: Rotors>10	No; 1 violation: WLOGP>5.88	No; 2 violations: XLOGP3>5, Heteroatoms<2	0.55
Guanosine	Yes: 0 violation	No; 1 violation: WLOGP>-0.4	No; 1 violation: TPSA>140	No; 1 violation: TPSA>131.6	No; 1 violation: TPSA>150	0.55
Quercetin	Yes; 0 violation	Yes	Yes	Yes	Yes	0.55
Medicinal Chemistry						
	PAINS	Brenk	Lead-likeness	Synthetic accessibility		
Phytol	0 alert	1 alert: isolated alkene	No; 2 violations: Rotors>7, XLOGP3>3.5	4.30		
Guanosine	0 alert	0 alert	Yes	3.85		
Quercetin	1 alert: catechol A	1 alert: catechol A	Yes	3.23		

4.4. DISCUSSION

Finding alternative treatments for multidrug-resistant bacteria is imperative hence hindering bacterial quorum sensing (QS) systems is an evolving approach to eradicating their pathogenicity than targeting their bactericidal effect (Wang, Zhao, et al., 2020). As a result, pharmaceutical industries and the scientific community are searching for plant-derived compounds like saponins, terpenoids, alkaloids, tannins, polyphenols and flavonoids, which are known to combat pathogens and improve the host's defense system (Chadha, Harjai & Chhibber, 2021). This study evaluated the minimum inhibitory concentrations of compounds and then assessed the selected plant extracts and compounds for their antibiofilm activities on MDR bacteria.

Based on the results, the preliminary screening of phytochemical compounds against selected MDR bacteria gave a clear indication of the compound's antibacterial activities. Compounds of guanosine and phytol showed significant antibacterial activities with MIC ranging between 0.016 – 0.063 mg/mL against the tested bacteria (Table 4.1). Noteworthy antibacterial activities for both guanosine and phytol revealed minimum inhibitory concentrations of 0.016 and 0.031 mg/mL against *S. pyogenes* and *S. aureus*, respectively. These two compounds of phytol (4.8%), diterpenoid alcohol and guanosine (22.3%), a purine nucleoside are identified from the methanolic extract of *Melianthus comosus* (Baloyi et al., 2021). Nocedo-Mena et al. (2019) reported the antibacterial activity of phytol against the clinical strain of methicillin-resistant *Staphylococcus aureus* (MRSA) (14-2095) with MIC = 0.2 mg/mL. In this study, phytol exhibited a MIC of 0.031 mg/mL against *Staphylococcus aureus* (ATCC 25923). The low MIC values suggest that a low concentration of the compounds is required for inhibiting the growth of the organism, hence drugs/compounds with lower MIC scores are more effective antimicrobial agents (Kowalska-Krochmal & Dudek-Wicher, 2021). According to Gibbons (2004) in Mamabolo et al. (2018), a MIC value of 0.064 mg/mL or lower is considered significant for individual phytochemicals. For this reason, both phytol and guanosine showed potent activities and qualify as potential antibacterial agents against the studied test pathogens.

Based on quantification of biofilm activity both in anti-adhesion (Table 4.2) and preformed biofilm (Table 4.3) steps, these extracts and compounds showed various results. The findings of this study indicated good inhibitory activity of the

plant extracts in the initial stage (anti-adhesion) of biofilm formation, especially for the GPB (*S. aureus* and *S. pyogenes*). Notably, compounds of guanosine and phytol revealed good anti-adhesion activity against *S. aureus*, *S. pyogenes* and *P. aeruginosa*, which performed better than ciprofloxacin and quercetin. This could be due to the ability of both extracts and compounds to restrict forces such as Van der Waals and electrostatic interaction that promote attachment to surfaces of the bacteria (Famuyide et al., 2019). As aforementioned, the GNB of *E. coli* and *K. pneumoniae* showed weak to no inhibitory activity of the plant extracts and compounds due to the three-component membrane being resistive to the diffusion of the extracts and compounds (Liu et al., 2019). Overall, the two phytochemical compounds significantly disrupted cell anti-adhesion and slightly reduced the performed biofilm biomass of the tested bacteria. The findings of this study signify that guanosine and phytol could play a role in preventing the cell-cell communication system

Several studies have proven that the eradication of biofilms is rather difficult as resistance was shown by various biofilm-forming microbes (Sarkar et al., 2015; Baloyi et al., 2019; Cosa et al., 2020). This could be because when biofilms are in their natural surroundings they include multiple species, whereby the inter-species may be the contributing factor to the growth and formation of this consortium of microorganisms (Giaouris et al., 2015). The mechanism of MDR bacteria is multi-factorial, thus this is not fully understood and requires further investigations (Guzzo et al., 2020). Furthermore, Giaouris et al. (2015) have indicated that the QS communication between bacteria in a biofilm has an impact on their relative resistance. The ability of extracts and compounds to reduce biofilm activity could be the result of the QS process of pathogenic bacteria by either impeding autoinducer synthesis or autoinducer reception and transduction or degrading autoinducers (Theodora, Dominika & Waturangi, 2019).

Further microscopic analysis using SEM and AFM was applied to visualize the morphological changes and surface morphologies of bacterial aggregation. The SEM analysis validated the biofilm inhibitory effect of guanosine and phytol and indicated a reduction in the biofilm aggregation of the *S. aureus*, *S. pyogenes* and *P. aeruginosa* (Figure 4.3). Surface morphology permits a key insight into bacteria compound interactions and allows better understanding into the developments of bacterial surface settlement, however, such studies are limited (Huang et al., 2015).

Similar to this study, Adeosun et al. (2022) indicated phytol significantly decreases the height and the roughness of *K. pneumoniae* using AFM.

The cytotoxicity of numerous natural products is likely to have adverse side effects on test subjects (Piccolella et al., 2021). As a result, it is crucial to establish whether plant extracts and secondary metabolites showing promising drug activities are effective in the tolerable toxicity and selectivity index range (Njeru & Muema, 2021). Famuyide et al. (2019) pointed out that *in vitro* cytotoxicity on cell lines cannot be compared to animal toxicity as several factors like gut interactions and bioavailability play a role. The study constitutes an *in vitro* proof-of-concept on the safety of the selected bioactive extracts and compounds. According to the results of the CCK-8 assay which determined the dose concentration required for Vero cell's viability and revealed that all extracts of *M. comosus* (methanol), *P. sidoides* (aqueous) and *V. karroo* (aqueous and methanol) had above 50% cell's viability at a 0.39 mg/mL except the aqueous extract of *M. comosus* (Table 1). Contrary to our study, Nyila et al. (2012) studied the cytotoxicity effect of *V. karroo* (ethyl acetate) extract against Vero cell using the XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assay, the extract had a cytotoxic effect with CC₅₀ value of 0.0455 mg/mL. This study has evaluated both aqueous and methanolic extract of *V. karroo* against Vero cells using the CCK-8 assay, which revealed a cytostatic effect with CC₅₀ values of 0.63 and 0.79 mg/mL, respectively.

Kudumela, McGaw & Masoko, (2018) mentioned that SI was applied to indicate the window between the cytotoxic and antibacterial activities of plant extracts at their inhibitory concentration. Overall, *V. karroo* cause much damage to the tested normal epithelial cells given at a relevant dose. SI was evaluated for each plant extract using the MIC for all the tested pathogens, this was to confirm that the antibacterial activities were associated with a specific ability to affect microbial growths and not purely linked to a general toxic effect. According to Elisha et al. (2017), a greater SI value of an extract frequently interprets that the biological activities are possibly not due to metabolic toxins. The LDH activity indicated that the tested plant extracts and compounds did not cause cell death even in a dose-dependent manner as presented in Table 4.6. These potentially studied plant extracts and compounds require to be completely investigated on other cell lines related to the traditional use in humans and performed *in vivo* assays, to verify their pharmacognostic and pharmaceutical significance (Asong et al., 2019). The

computational tool applied in this study is one the most commonly used, to sieve compounds with unsuitable properties, particularly poor absorption, distribution, metabolism, excretion, and toxicity (ADMET) profiles (Tian et al., 2015). The virtual screening of the drug-likeness characteristics of the studied compounds provided significant predictions required to select the suitable compounds which have favourable ADME properties. The drug-likeness screening of compounds revealed that guanosine and quercetin obeyed Lipinski's rule, with phytol having one violation. Lipinski suggested the well-known drug-likeness filter the "Rule of Five", which states that for a drug to be orally absorbed it should adhere to these provided rules of the molecular weight (MW) ≤ 500 , octanol/water partition coefficient (logP) ≤ 5 , number of hydrogen bond donors ≤ 5 and number of hydrogen bond acceptors ≤ 10 (Lipinski et al., 1997; Tian et al., 2015), except for a drug having one violation. Deficient pharmacokinetic properties are deemed as one of the major reasons compounds fail during the drug development process (Matias et al., 2017). Findings from this study, gave an insight about the structural features of the chemical compounds which are imperative from the pharmacokinetic prospect for additional hit-to-lead development (Matias et al., 2017).

4.5. CONCLUSION

The findings of this chapter demonstrated the promising biofilm inhibition of bioactive extracts and compounds that can improve the discovery of antibiofilm agents and can assist with impeding bacterial diseases, without contributing to the antimicrobial resistance crisis. Notably, *M. comosus*, *P. sidoides* and *V. karroo* have demonstrated good anti-adhesion activity against GPB (*S. aureus* and *S. pyogenes*). Guanosine and phytol also revealed good antibacterial and antibiofilm activities against the tested bacteria. Thus, the results reported propose that the two compounds demonstrated their potential with a novel mechanism of biofilm inhibition. Furthermore, these compounds could potentially be used to eliminate the production of other virulence factors in pathogenic bacteria. Potentially this provides an important advancement in discovering a treatment for infectious diseases and further highlights compounds that qualify to be used as antibiofilm agents to combat several MDR bacteria as demonstrated by their potential to target bacterial QS.

This study also highlighted the *in vitro* proof-of-concept of the tested plant extracts and compounds had a cytostatic instead of cytotoxic effect on Vero cells within a specific dose. Guanosine and phytol showed no toxic effects on both the bacterial cells and mammalian cells. These compounds indicated that they can be used safely on epithelial with no malignant cells without causing cell damage. Furthermore, the drug-likeness prediction scores of the compounds demonstrated they could be potentially used in the early stages of drug discovery as antipathogenic compounds. Understanding the molecular mechanisms of the compound's biological activity is crucial, particularly if these compounds are to be further explored for other purposes. Thus, providing a distinct overview of the molecular mechanism is important.

In the next chapter, investigating the molecular mechanism of selected compounds for their quorum sensing inhibition through an *in-silico* approach was performed using *P. aeruginosa* as the GNB model organism.

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CHAPTER FIVE

***IN SILICO* SCREENING OF ANTIPATHOGENIC PROPERTIES OF PHYTOCHEMICAL COMPOUNDS USING *PSEUDOMONAS AERUGINOSA* AS A MULTI-DRUG RESISTANT MODEL ORGANISM**

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5.1. INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative opportunistic human pathogen that causes acute and chronic infections, especially in immunocompromised individuals and poses a fatal hazard to hospitalised patients (Singh & Bhatia, 2018; Malgaonkar & Nair, 2019). The pathogenicity and colonisation of *P. aeruginosa* on host tissues, surfaces or medical devices cause nosocomial infections such as those of the urinary tract, cystic fibrosis and wounds (Cosa et al., 2020). This pathogen triggers biofilm formation and several virulence genes in a cell density-dependent manner through a bacterial communication system, well accepted as quorum-sensing (QS) (Kostylev et al., 2019). The world health organization (WHO) ranks *P. aeruginosa* as 1st priority pathogen, which is critical and urgently requires the development of new therapeutic options¹². Since *P. aeruginosa* is also part of the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp), it serves as a multi-drug resistant (MDR) model organism for the development of antivirulence drugs targeting QS (Soukarieh, Williams, Stocks and Camara, 2018)

P. aeruginosa has four known QS systems: LasI/LasR, RhII/RhIR, *Pseudomonas* quinolone signalling (PQS) and integrated quorum signalling (IQS) (Cosa, Rakoma, Yusuf and Tshikalange, 2020). The Las system consists of a transcriptional activator (LasR) that activates LasI synthase to produce autoinducers N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) (Pena et al., 2019). The Rhl system consists of RhIR which activates RhII synthase to produce N-butyryl-L-homoserine lactone (C4-HSL) (Wang, Zhao, et al., 2020). The PQS system acts as a co-inducer for the transcriptional stimulator PqsR and produces 2-heptyl-3-hydroxy- 4-quinolone that is chemically unique from the autoinducer signal molecules of the Las and Rhl systems (Ueda and Wood, 2009). Lastly, the IQS structurally established the 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde molecule (Malgaonkar and Nair, 2019). The four cell-communication systems are interconnected and regulated hierarchically, triggering several virulence factors,

¹² <https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed>

formation of biofilms and antibiotic resistance (Lee and Zhang, 2015), thus necessitating novel approaches for combating *P. aeruginosa* infections.

Quorum sensing inhibition (QSI) strategies promise to combat MDR bacteria due to their ability to regulate pathogenicity and virulence (Alvarez et al., 2014). Disruption of QS is a suitable strategy possibly due to its mechanism of action focusing on hindering the formation of biofilms and other virulence factors causing infection, without imposing selective pressure on the pathogen. Selective pressure is regarded as a key cause of bacterial resistance (Singh & Bhatia, 2018).

Medicinal plant decoctions have been investigated for their biologically active compounds as a source of antibacterial drugs since time immemorial. Currently, medicinal plants are receiving attention to aid a search for secondary metabolites with the potential to act against pathogens and prevent QS communication (Khan et al., 2018). However, there is a paucity of knowledge about their ability to disarm QS or as antipathogenic agents. South Africa has a rich diversity of medicinal plants that are used as traditional remedies to treat various diseases in both humans and livestock (Maroyi, 2019).

Melianthus comosus, *Pelargonium sidoides* and *Vachellia karroo* were the plants of interest due to their potent activity; and to the best of our knowledge, there are no reports on the *M. comosus*, *P. sidoides* and *V. karroo* phytochemicals with their ability to competitively bind to *P. aeruginosa* QS receptor protein and interfere with the activity of acyl-homoserine lactone (AHL) molecules.

In the previous chapter, selected suitable compounds with their antagonistic effect against the CviR protein of the antiquorum sensing (AQS) screening model organism *C. violaceum* were revealed. Hence in this chapter, the same selected phytochemicals were employed to further explore their QSI/AQS potential for proficient activity and binding affinity predictions against LasR and PqsR protein's active sites of MDR *P. aeruginosa* as a model pathogen.

5.2. MATERIALS AND METHODS

5.2.1. *In silico* modelling of selected active compounds against LasR and PqsR proteins

Molecular docking (MD) analysis for *P. aeruginosa* was performed as described in section 3.2.2 with minor modifications. Here, the two QS receptor proteins included LasR and PqsR of *P. aeruginosa* ATCC 9721 (PDB: 2UV0 and 4JVD). The crystal structures of the other two receptor proteins (RhlR and IQS) of *P. aeruginosa* have not yet been determined (Wang, Zhao, et al., 2020), hence could potentially be explored for future investigations.

5.2.2. Molecular dynamics simulations for the best-docked ligands

A similar molecular dynamics simulations (MDS) procedure was also applied for the best-docked ligands including reference compounds for both targeted proteins of 2UV0 and 4JVD as described in section 3.2.3.

5.3. RESULTS

5.3.1. *In silico* modelling of phytochemical compounds

The same compounds evaluated for CviR' protein were targeted on the LasR and PqsR proteins. These included α -D-glucopyranoside, methyl, 1,2,3-benzenetriol, 2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one, decanedioic acid, guanosine, linalool, neophytadiene, phytol, pyroglutamic acid and sucrose where their binding potential to the two QS receptor proteins of *P. aeruginosa* were studied. Both these proteins trigger various virulence phenotypes. For example, the LasR protein regulates LasA protease, LasB elastase and exopolysaccharides while the PqsR protein controls the pyocyanin, hydrogen cyanide and LecA lectin (Guzzo et al., 2020). The binding affinities of the selected compounds are summarised in Table 5.1 and ligand interactions only of the best docked ligand-protein complex of 2UV0 and 4JVD are presented in Figures 5.1 and 5.2, respectively.

Sucrose was the most antagonistic compound for both 2UV0 and 4JVD receptors which showed docking scores of -10.424 kcal/mol with glide energy of -33.109 kcal/mol and -9.985 kcal/mol with glide energy of -25.808 kcal/mol for 2UV0 and 4JVD, respectively. Guanosine followed by showing MD scores of -8.376 kcal/mol with glide energy of -49.614 and -9.133 kcal/mol with -41.24 kcal/mol for 2UV0 and 4JVD, respectively. The compounds, α -D-glucopyranoside, methyl, 1,2,3-benzenetriol, phytol and 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one also showed good MD scores of -7.251 kcal/mol, -6.463 kcal/mol, -5.664 kcal/mol and -5.358 kcal/mol, respectively for 2UV0 whereas linalool, neophytadiene and pyroglutamic acid showed weaker binding affinities as presented in Table 5.1. Sucrose was found to be potent in binding compared to the reference compound quercetin (-9.946 kcal/mol) and the native ligand 3-oxo-C12-homoserine lactone with a score of -9.803 kcal/mol (Table 5.1).

Figure 5.1 presented the interaction networks between the best-docked ligands of 2UV0. Thirteen hydrophobic interactions and hydrogen bonding with Thr75, Val76 and Leu125 were formed for sucrose (Figure 5.1A) whereas guanosine formed 14 hydrophobic interactions, hydrogen bonding with Tyr47, Arg61, Thr75 and π - π staking with Tyr64 (Figure 5.1B). An α -D-glucopyranoside, methyl had 16 hydrophobic interactions and formed hydrogen bonds with Try56, Trp60, Leu110 and Tryp93 (Figure 5.1C), followed by 1,2,3-benzenetriol forming 15 hydrophobic interactions and binding to hydrogen bonding to Thr75, Ser129 and π - π staking interaction with Tyr56 (Figure 5.1D) while phytol formed 28 hydrophobic interactions and forming hydrogen bonding with Thr75 (Figure 5.1E). A 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one formed 10 hydrophobic interactions and hydrogen bonds with Tyr56, Ser129, Trp60 (Figure 5.1F). Quercetin formed hydrogen bonds with Ser155, Asp97 and Met135, π - π staking with Tyr88 and Tyr 80 (Figure 3.3G), while 3-oxo-C12-homoserine lactone hydrogen bonded with Tyr56, Ser129, Trp60 and Asp73 (Figure 3.3H). Cinnamaldehyde formed hydrogen bonds with Tyr56, and Ser129 and had 10 hydrophobic interactions (Figure 3.3I). Cinnamaldehyde, 3-oxo-C12-homoserine lactone and 1,2,3-benzenetriol formed hydrogen bonding with Ser129.

Table 5.1: Molecular docking results of selected compounds on the 2UV0 and 4JVD

2UV0			
Compound names	Docking score	Glide energy	Interactions
α -D-Glucopyranoside, methyl	-7.251	-31.39	Try56, Tyr93, Trp60, Leu110
1,2,3-Benzenetriol	-6.463	-25.597	Tyr93, Thr75
2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	-5.358	-33.244	Tyr56, Trp60, Ser129
Decanedioic acid	-3.485	-37.51	Ser129
Guanosine	-8.376	-49.614	Tyr47, Tyr64, Thr75, Arg61
Linalool	-4.556	-22.102	Thr75
Neophytadiene	-3.232	-37.941	Nil
Phytol	-5.664	-31.858	Thr755
Pyroglutamic acid	-3.169	-22.138	Tyr56, Ser129, Asp73
Sucrose	-10.424	-33.109	Thr75, Val76, Leu125
Reference compounds			
3-oxo-C12-HSL	-9.803	-61.317	Tyr56, Trp60, Ser129, Arg61, Asp73
Cinnamaldehyde	-5.275	-24.305	Tyr56, Ser129
Quercetin	-9.946	-45.128	Thr75
4JVD			
α -D-Glucopyranoside, methyl	-7.750	-30.688	Leu197, Leu207, Leu208, Ile236
1,2,3-Benzenetriol	-6.846	-26.504	Leu207, Leu208
2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	-5.552	-24.809	Leu197, Leu208
Decanedioic acid	-3.365	-28.365	Gln194
Guanosine	-9.133	-41.24	Leu197, Ile195, Ile236, Gln194
Linalool	-4.289	-25.796	Leu197, Leu208
Neophytadiene	-4.946	-27.453	Nil
Phytol	-7.792	-34.167	Ile195, Ile236
Pyroglutamic acid	-5.117	-23.561	Ile236, Gln194
Sucrose	-9.985	-25.808	Leu197, Leu207, Gln194, Ser196, Arg209
Reference compounds			
2-heptyl-3-hydroxy-4-quinolone	-7.301	-34.71	Leu197
Cinnamaldehyde	-6.182	-24.442	Leu197
Quercetin	-11.448	-45.133	Leu197, Ile236, Arg209, Thr265

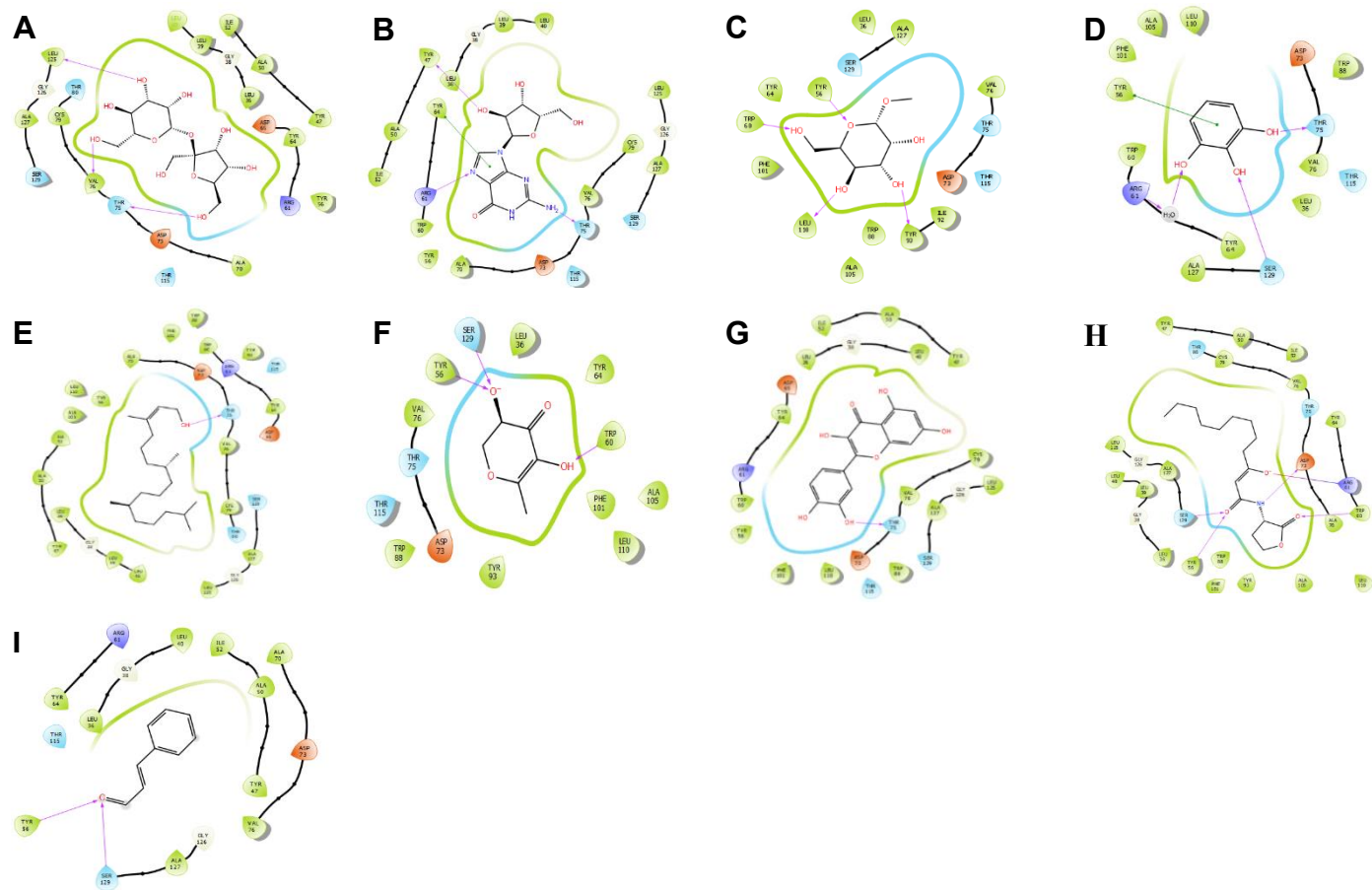


Figure 5.1: Interaction network between 2UV0 protein and the active potential QSI compounds. The protein residues with a negative charge are shown in red, positive charge in velvet, polar in cyan, and hydrophobic in parrot green. The H-bond interactions are shown as a purple arrow, pi-pi stacking as a green line. (A) Sucrose; (B) Guanosine; (C) α -D-glucopyranoside, methyl; (D) 1,2,3-Benzenetriol; (E) Phytol; (F) 2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one; (G) Quercetin; (H) 3-oxo-C12-HSL and (I) Cinnamaldehyde.

Based on the findings, phytol, α -D-glucopyranoside, methyl, 1,2,3-benzenetriol, 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one and pyroglutamic acid showed good binding affinities to the 4JVD protein with docking scores of -7.792 kcal/mol, -7.750 kcal/mol, -6.846 kcal/mol, -5.552 kcal/mol and -5.117 kcal/mol, respectively. The positive control of quercetin was shown to be the best reference compound with the highest score of -11.448 kcal/mol and glide energy of -45.133 kcal/mol binding to 4JVD protein, which even bound better than the native ligand of 2-heptyl-3-hydroxy-4-quinolone that had a score of -7.301 kcal/mol with glide energy of -34.71 kcal/mol whilst cinnamaldehyde showed a score of -6.182 kcal/mol and glide energy of -24.442 kcal/mol.

Concerning the 4JVD protein interaction network, sucrose showed hydrogen bonds with residues of Leu197, Leu207, Arg209, Gln194, and Ser196 (Figure 5.2A), followed by guanosine forming hydrogen bonds with Leu197, Ile236, Gln194 and Ile195 (Figure 5.2B) while α -D-glucopyranoside, methyl formed hydrogen bonds with Leu197, Leu207, Leu208 and Ile236 (Figure 5.2C) and phytol formed hydrogen bonds with Ile195 and Ile236 (Figure 5.2D). A 1,2,3-benzenetriol formed hydrogen bonds with Leu207 and Leu208 followed by 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one that formed hydrogen bonds with Leu197 and Leu208 and pyroglutamic acid formed hydrogen bonds with Ile236 and Gln194 as presented in Figure 5.2E, Figure 5.2F and Figure 5.2G, respectively. Quercetin showed formed hydrogen bonds with Leu197, Thr265, Ile236 and Arg209 (Figure 5.2H). Both the native ligand 2-heptyl-3-hydroxy-4-quinolone and cinnamaldehyde formed a hydrogen bond with Leu197 as shown in Figure 5.2I and Figure 5.2J, respectively.

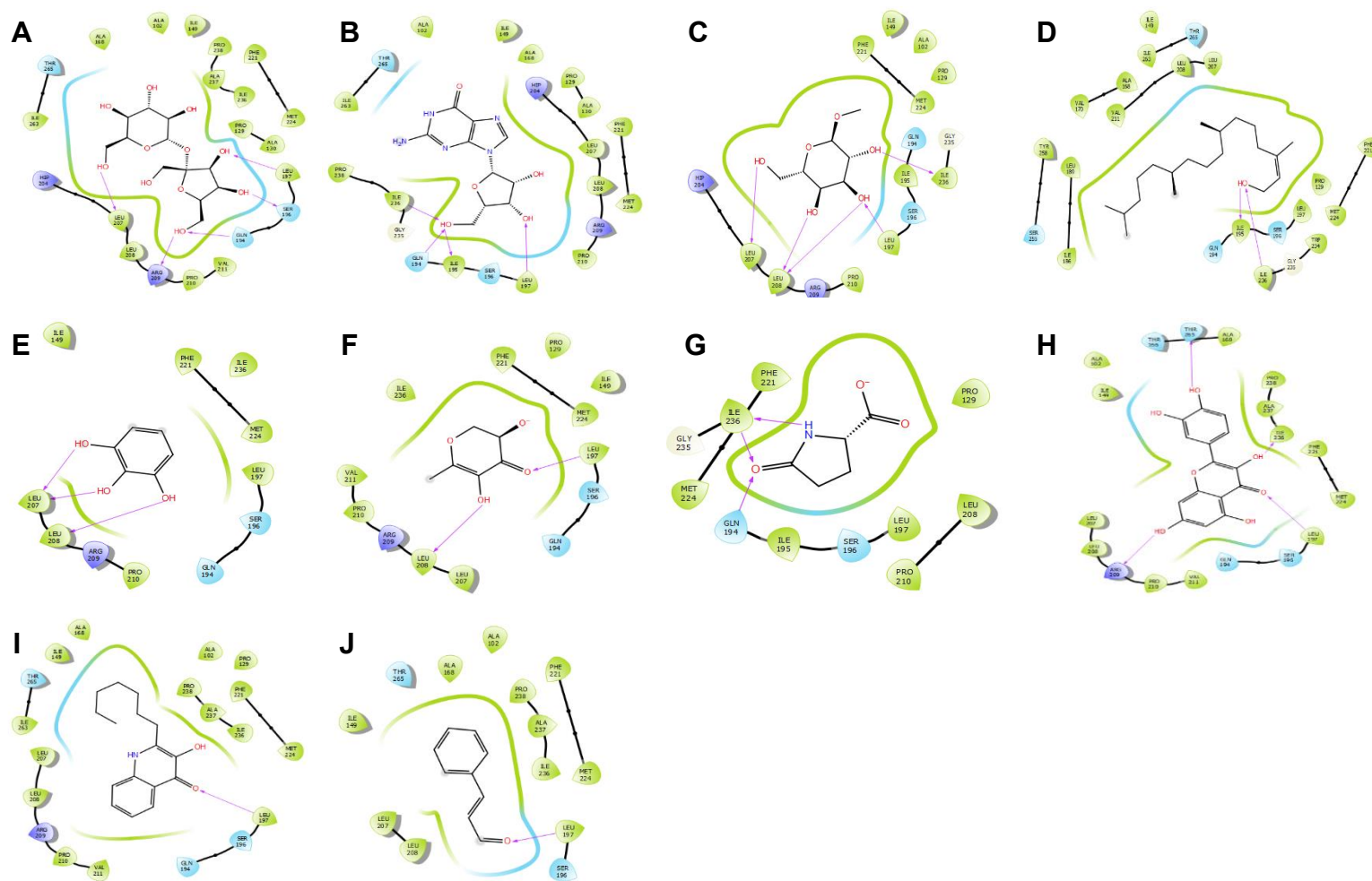


Figure 5.2: Interaction network between 4JVD protein and the active potential QSI compounds. The protein residues with a negative charge are shown in red, positive charge in velvet, polar in cyan, and hydrophobic in parrot green. The H-bond interactions are shown as a purple arrow, pi-pi stacking as a green line. (A) Sucrose; (B) Guanosine; (C) α -D-glucopyranoside, methyl; (D) Phytol; (E) 1,2,3-Benzenetriol; (F) 2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one; (G) Pyroglutamic acid; (H) Quercetin; (I) 2-heptyl-3-hydroxy-4-quinolone and (J) Cinnamaldehyde.

5.3.2. Molecular dynamics simulations

5.3.2.1 Structural stability and flexibility of 2UV0 and 4JVD proteins

The MDS results for best-docked ligands of sucrose, guanosine, α -D-glucopyranoside, methyl, phytol and 1,2,3-benzenetriol are presented as the root mean square deviation (RMSD) and root mean square fluctuation (RMSF) of the C- α backbone atoms with respective crystal structures 2UV0 and 4JVD in Figure 5.3. The 2UV0 bound and unbound systems convergence was reached early in the simulation at approximately 5 ns and was followed by steady atomic motions in all systems until 20 ns, where there was an observed separation of atomic motions in some of the bound systems notably the 1, 2, 3-benzenetriol (red) and guanosine (blue) bound systems as presented in Figure 5.3A. This separation of atomic motions was characterized by a slight increase in deviations of the c- α atoms in the backbone of 2UV0. This deviation continued until the ~30 ns mark where atoms assumed steady motions till the end of the simulation as presented in Figure 5.3A. In Figure 5.3A phytol (cyan) showed an average RMSD value of 1.47Å indicating a good solution for 2UV0 better than the reference compounds cinnamaldehyde (1.54Å) in yellow and quercetin (1.69Å) in mustard. The least stable solutions were observed with 1, 2, 3-benzenetriol and guanosine bound systems with average RMSD values of 1.76 Å and 1.70 Å respectively as shown in Figure 5.3A. Comparatively, all 4JVD systems had stabilized except for 1,2,3-benzenetriol (red), α -D-glucopyranoside (green) and phytol (cyan) with corresponding high average RMSD values of 3.06Å, 3.49Å and 3.07Å, respectively. While the systems of Apo (black), guanosine (blue), sucrose (purple), cinnamaldehyde (yellow) and quercetin (mustard) showed RMSD averages of 2.79Å, 2.10Å, 2.27Å, 2.74Å and 2.19Å respectively as presented in Figure 5.3B. The average RMSF values estimated for the 2UV0 systems were 12.04Å, 11.83Å, 11.28Å, 11.54Å, 11.34Å, 7.49Å, 12.99Å and 8.96Å for the Apo, 1,2,3-benzenetriol, α -D-glucopyranoside, methyl, guanosine, phytol, sucrose, cinnamaldehyde and quercetin, respectively (Figure 5.3C). The bound and unbound systems of 4JVD showed RMSF averages of 9.63Å, 13.56Å, 30.18Å, 11.69Å, 30.19Å, 13.20Å, 11.84Å and 15.95Å for the Apo, 1,2,3-Benzenetriol, α -D-glucopyranoside, methyl, guanosine, phytol, sucrose, cinnamaldehyde and quercetin systems, respectively as presented in Figure 5.3D.

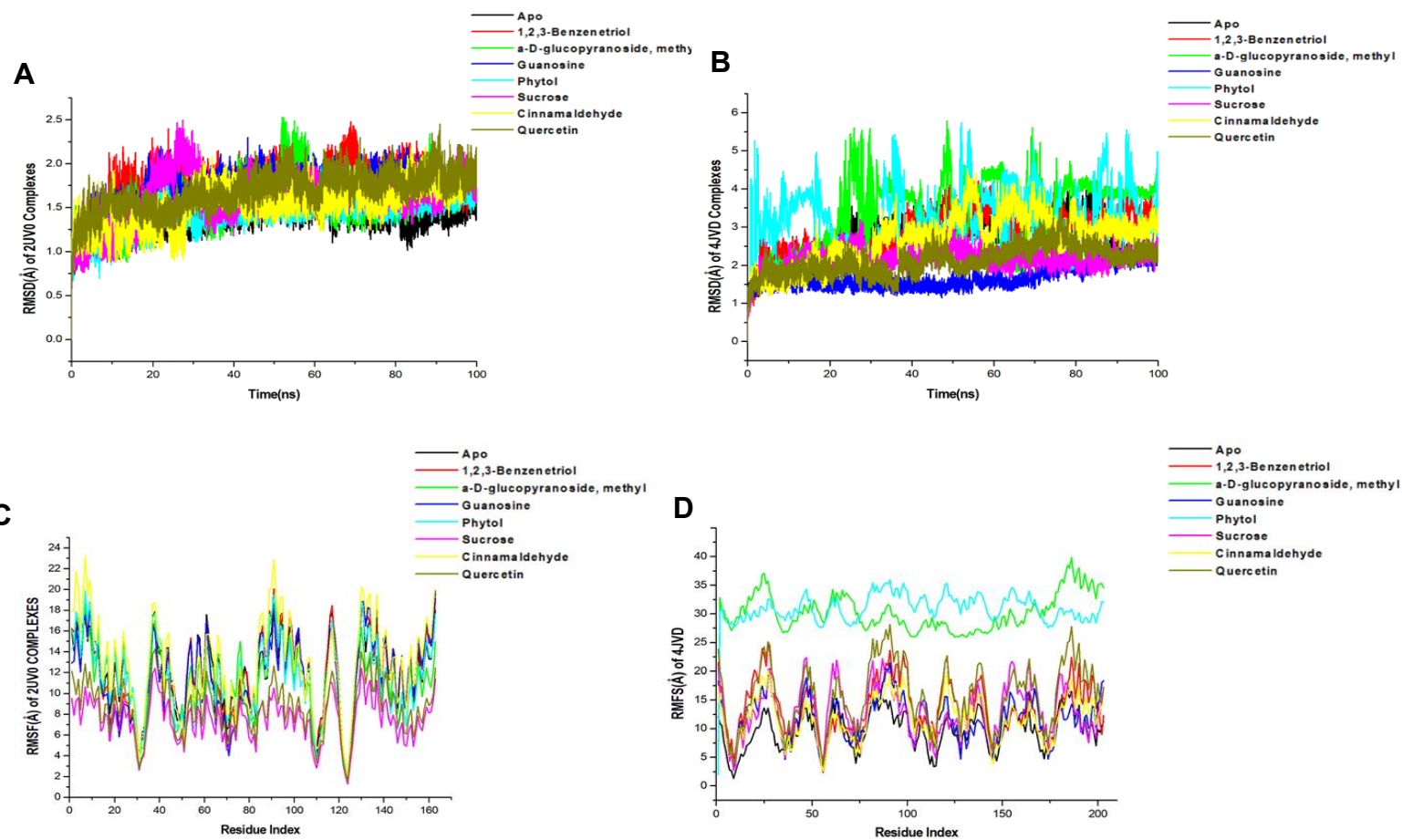


Figure 5.3: Trajectory analysis of 100 ns simulation for the unbound and bound systems of phytochemical compounds for C- α root-mean-square deviation (RMSD) plots of quorum sensing proteins: (A) 2UV0 and (B) 4JVD; C- α root-mean-square-fluctuation (RMSF) plots: (C) 2UV0 and (D) 4JVD.

5.3.2.2 Differential binding free energy analysis

All the test phytochemical compounds showed varying total binding free energies (ΔG_{bind}) (Table 5.2). Bound systems of 1,2,3-benzenetriol, α -D-glucopyranoside methyl, guanosine, phytol, sucrose, cinnamaldehyde and quercetin bound to 2UV0 with binding energies of -22.68 kcal/mol, -27.89 kcal/mol, -41.67 kcal/mol, -55.85 kcal/mol, -47.02 kcal/mol, -16.89 kcal/mol and -38.13 kcal/mol, respectively. Whereas for the 4JVD bound complexes, the total free energies were estimated for 1,2,3-Benzenetriol, α -D-glucopyranoside methyl, guanosine, phytol, sucrose, cinnamaldehyde and quercetin to be -31.40 kcal/mol, -22.10 kcal/mol, -27.98 kcal/mol, -34.22 kcal/mol, -14.08 kcal/mol, -17.18 kcal/mol and -25.65 kcal/mol respectively.

Table 5.2: Binding free energy estimates for the simulated complexes.

2UV0					
Compound names	ΔE_{vdw}	ΔE_{ele}	ΔG_{gas}	ΔG_{sol}	ΔG_{bind}
α -D-glucopyranoside, methyl	-27.70 $\pm$ 0.17	-31.75 $\pm$ 0.21	-59.45 $\pm$ 0.22	31.56 $\pm$ 0.15	-27.89 $\pm$ 0.19
1,2,3-Benzenetriol	-14.35 $\pm$ 0.25	-30.93 $\pm$ 0.66	-45.29 $\pm$ 0.50	22.61 $\pm$ 0.25	-22.68 $\pm$ 0.36
Guanosine	-42.57 $\pm$ 0.19	-43.47 $\pm$ 0.23	-86.04 $\pm$ 0.34	44.37 $\pm$ 0.22	-41.67 $\pm$ 0.20
Phytol	-59.69 $\pm$ 0.22	-8.12 $\pm$ 0.28	-67.81 $\pm$ 0.30	11.97 $\pm$ 0.15	-55.85 $\pm$ 0.24
Sucrose	-44.12 $\pm$ 0.24	-49.10 $\pm$ 0.50	-93.22 $\pm$ 0.41	46.20 $\pm$ 0.22	-47.02 $\pm$ 0.29
Cinnamaldehyde	-23.40 $\pm$ 0.13	-15.07 $\pm$ 0.24	-38.48 $\pm$ 0.28	21.59 $\pm$ 0.22	-16.89 $\pm$ 0.15
Quercetin	-42.95 $\pm$ 0.18	-28.89 $\pm$ 0.52	-71.84 $\pm$ 0.50	33.70 $\pm$ 0.31	-38.13 $\pm$ 0.28
4JVD					
α -D-glucopyranoside, methyl	-27.74 $\pm$ 0.18	-15.73 $\pm$ 0.42	-43.48 $\pm$ 0.45	21.38 $\pm$ 0.25	-22.10 $\pm$ 0.24
1,2,3-Benzenetriol	-17.07 $\pm$ 0.20	-42.67 $\pm$ 0.53	-59.74 $\pm$ 0.50	28.34 $\pm$ 0.28	-31.40 $\pm$ 0.26
Guanosine	-38.00 $\pm$ 0.21	-13.98 $\pm$ 0.61	-51.18 $\pm$ 0.55	23.20 $\pm$ 0.48	-27.98 $\pm$ 0.23
Phytol	-32.34 $\pm$ 1.31	-7.95 $\pm$ 0.41	-40.28 $\pm$ 1.65	6.06 $\pm$ 0.29	-34.22 $\pm$ 1.39
Sucrose	-21.93 $\pm$ 0.27	-15.03 $\pm$ 0.53	-36.96 $\pm$ 0.50	22.88 $\pm$ 0.34	-14.08 $\pm$ 0.26
Cinnamaldehyde	-20.89 $\pm$ 0.14	-3.53 $\pm$ 0.27	-24.42 $\pm$ 0.33	7.24 $\pm$ 0.24	-17.18 $\pm$ 0.17
Quercetin	-33.70 $\pm$ 0.19	-8.20 $\pm$ 0.30	-41.91 $\pm$ 0.33	16.26 $\pm$ 0.22	-25.65 $\pm$ 0.18

ΔE_{ele} = electrostatic energy; ΔE_{vdW} = van der Waals energy; ΔG_{bind} = total binding free energy;

ΔG_{sol} = solvation free energy ΔG = gas-phase free energy.

5.4. DISCUSSION

The significance of expanding various coherent drug design procedures involving *in-silico* assessment in finding new drugs can provide an insight into the chemical components that show ligand-protein network interactions pertinent to the QS protein targets (Ramírez & Caballero, 2018). Studying the antivirulence compounds for *P. aeruginosa* required the exploration of the probable connections between the test compounds and the ability to impede LasR and/or PqsR system, activated by its signal molecule 3-oxo-C12-HSL, that binds to the LasR (2UV0) protein while 2-heptyl-3-hydroxy-4-quinolone binds to the PqsR (4JVD) protein with their effects on the production of rhamnolipids, biofilms and pyocyanin (Hossain *et al.*, 2020). Here, the *in-silico* modelling was undertaken to pin the compounds responsible for the efficacy of *M. comosus* extracts against *P. aeruginosa* with their mode of action. Furthermore, to understand the binding affinities on the two QS receptor proteins LasR and PqsR.

Based on the findings, *M. comosus* extracts had sucrose, guanosine, phytol and 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one as the most dominant compounds, binding to 2UV0 and 4JVD proteins. Guanosine, phytol, pyroglutamic acid and 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one including positive controls revealed improved docking scores with varying glide energies (Table 5.1). Sucrose displayed a higher docking score as compared to the reference compounds whereas guanosine exhibited a docking score closer to both quercetin and 3-oxo-C12-HSL. Similar to our study, Kumar *et al.* (2015) demonstrated that 3-oxo-C12-HSL showed a docking score of -9.0 kcal/mol for the 2UV0 protein. A report by Singh and Bhatia (2018) confirmed results obtained here with quercetin molecule to compete with the natural ligand to bind to the LasR protein. Conversely, cinnamaldehyde had lower scores though it has been reported in the literature (Topa *et al.*, 2020) as a QSI agent particularly for *in vitro* assays.

When docking PqsR protein, results revealed sucrose, guanosine and phytol showing better binding scores as compared to the native ligand 2-heptyl-3-hydroxy-4-quinolone, whereas the reference compound cinnamaldehyde had a lower docking score (Table 5.1). Congruent to this study, Wang *et al.* (2020) have shown a compound's potential to bind better to the LasR and PqsR protein as compared to the native ligands. Similarly, Annapoorani *et al.* (2012) also revealed various

compounds had higher docking scores than the natural molecules. There were several amino acid residues which showed similarities in the binding interactions between the selected compounds and native ligands. Sucrose, guanosine and 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one all formed H-bond with Leu197 residue which indicates that these compounds' residues bind to the key active site of the receptor, as the native ligand was also shown to form H-bond with Leu197 (Figure 5.2). Furthermore, the findings also revealed, that 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one and 3-oxo-C12-HSL formed hydrogen bond with Ser129, Tyr56 and hydroxyl group with Trp60 for the 2UV0 protein. This suggests that these compounds can compete with the native ligand and bind to the protein's active pocket. In contrast, linalool, decanedioic acid and neophytadiene had weaker binding affinities towards the 2UV0 and 4JVD proteins. This is because these are long carbon chain compounds, they serve as inactive compounds as per the criteria of choosing potential AHL antagonists is concerned.

The best-docked ligands: sucrose, guanosine, 1,2,3-benzenetriol, α -D-glucopyranoside, methyl and phytol identified from MD were selected for further study on MDS and binding free-energy calculations. MDS gives a clear understanding of the actual movement of the protein-ligand systems over time (Vieira et al., 2022). According to the results, phytol appeared to be more stable among the compounds upon binding to 2UV0 lower than the rest, including cinnamaldehyde and quercetin (QSI controls). It is estimated that the lower the RMSD value, the more stable the system. All the studied compounds in complex with 2UV0 maintained steady atomic motions from the 5 ns mark to the end of the 100 ns simulation run as evidenced by the plateau shape in the plots (Figure 5.3A). The stability of all systems at the 100 ns mark, highlighted the reliability of the findings with phytol being the most stable compound bound to the 2UV0 protein, in contrast, guanosine was the most stable for the 4JVD protein. The stability of guanosine with the 4JVD protein correlates with MD results because the docking score was higher than that observed for 2UV0 protein.

In overall essence, phytol bound better to the two QS receptor proteins of *P. aeruginosa* as compared to the biomonitor strain, *C. violaceum*. The assessment for the stability of the bound and unbound states of the compounds for 4JVD showed similar outcomes for 2UV0.

In most conformational studies, a higher RMSF value correlates to a more flexible structure, and a lower value correlates to a less flexible or rigid conformation.

The phytochemical compounds bound complexes showed lower RMSF averages in contrast to the Apo, suggesting the binding of these compounds to the 2UV0 results in a less flexible conformation. Also, cinnamaldehyde and quercetin had a varying effect on the conformation of 2UV0 which was characterized by high residual flexibility (more flexible structure) in the cinnamaldehyde (12.99Å) system in contrast to the Apo while fluctuations were lower in the quercetin (8.69Å) system. This substantial impact on the flexibility of 2UV0 as enacted by the phytochemical compounds could interfere with the normal biological activity of the QS 2UV0 target and thus may have a therapeutic impact. Relatively, the binding of all compounds including the reference compounds resulted in a more flexible 4JVD conformation evidenced by the high average RMSF values computed. α -D-glucopyranoside, methyl (30.18Å) and Phytol (30.91Å) showed to be more flexible than the other compounds.

These results suggest the binding of the compounds to the proteins are spontaneous with minimal kinetic hindrance (Souza et al., 2020). Again, the van der Waals, electrostatic and gas-phase energies contributed significantly to the compounds' binding and stability (Zhang, Pérez-Sánchez & Lightstone, 2015). Altogether, the compounds showed good total free binding energies to the two targeted QS proteins further highlighting their potential therapeutic effect. These phytochemical compounds demonstrated similar binding effects with known QSI quercetin and cinnamaldehyde. Results from the *in-silico* study revealed interpretation of structure-function relationships amongst the test compounds and known quorum sensing inhibitors, which supports their potential use in combating bacterial virulence and quorum sensing activity. Although, sucrose displayed a high MD score for both proteins (LasR and PqsR), was excluded due to the negative connotation of sugar as a potential drug candidate.

Phytol was potent among the compounds due to its relatively more stable impact on the structure and function of the targets coupled with the formation of a stronger binding affinity and engagement in more crucial interactions. Guanosine also revealed MD scores closer to quercetin and can be ascribed to common functional groups of both structures. Their structures both contain a hydroxyl group, a functional group with the chemical formula -OH that is made up of one oxygen

atom covalently bound to one hydrogen atom. Additionally, guanosine is composed of guanine which has a carbonyl group, a functional group composed of a carbon atom double-bonded to an oxygen atom: C = O which is also present in the structure of 3-oxo-C12-HSL and quercetin. These structural similarities can pose a similarity in their docking scores. Therefore, guanosine was found to potentially act as an antagonist of 2UV0 and 4JVD proteins, this compound was thus chosen for further *in vitro* studies together with the extracts.

5.5. CONCLUSION

In-silico modelling provided an insight into identifying novel compounds capable of inactivating or activating regulator proteins in *P. aeruginosa*. Five compounds were screened to determine their proficient activity and binding affinities on 2UV0 and 4JVD protein, with sucrose, guanosine, phytol 1,2,3-benzenetriol and α -D-glucopyranoside, methyl showing significant binding affinities. These compounds showed improved docking scores indicating their stability of protein-ligand complexes and hydrogen bond interactions. Furthermore, the compounds showed their impact on the structural and functional activities through stabilizing and increasing flexibility in the targeted proteins. The compounds also engaged in high-affinity interactions with key residues in the binding sites of the targets which resulted in strong binding energies. Guanosine was the best compound that had the antagonistic activity against the two receptor proteins. Thus, the validation of these *in-silico* findings of the guanosine to hinder the virulence factors of *P. aeruginosa* is required. The succeeding chapter investigated the *in-vitro* studies of the potential guanosine and *M. comosus* extracts for their antivirulence properties on the MDR model pathogen *P. aeruginosa*.

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CHAPTER SIX

IN VITRO* VALIDATION OF ANTIVIRULENCE PROPERTIES OF *MELIANTHUS* *COMOSUS* (VAHL) AGAINST *PSEUDOMONAS AERUGINOSA

Parts of this chapter have been published as Baloyi IT, Adeosun IJ, Yusuf AA, Cosa S (2021). *In silico* and *in vitro* screening of antipathogenic properties of *Melanthus comosus* (Vahl) against *Pseudomonas aeruginosa*. *Antibiotics*, 10 (679): 1–23. <https://doi.org/10.3390/antibiotics10060679>.

6.1. INTRODUCTION

The pathogenicity of *P. aeruginosa* depends on its ability to trigger numerous virulence factors regulated by the QS gene expression mechanism (Azuama et al., 2020). Virulence describes the potential of a pathogen to infect and/or harm a host by tackling their immune system and colonising, this impels the severity of the infection (Chadha, Harjai & Chhibber, 2021). *P. aeruginosa* virulence factors contribute to acute and chronic infections including virulence phenotypes such as pyocyanin, which aids in immune evasion, LasA protease, which disrupts the epithelial barrier, LasB proteases, which degrade matrix proteins, rhamnolipids, which cause immune cell necrosis, and LecA lectin, which promotes bacterial colonization (Pattnaik et al., 2018). The production of pyocyanin signifies one of the crucial virulence factors, which causes oxidative stress whereas the various kinds of motility performs a key part in microbial settlement on surfaces and the development of biofilms (Murray, Ledizet & Kazmierczak, 2010; Pejčić et al., 2020).

Currently, the antivirulence strategy is one of the widely studied approaches because it targets the specific virulence factors involved within the pathogen rather than the bacterial viability (Azuama et al., 2020). Phytochemical that have antibacterial properties could be contributing to their QS inhibition (Vattem et al., 2007). Thus, the discovery of antipathogenic agents such as quorum sensing inhibitors (QSI) from natural products such as phytochemical compounds could be utilised for the treatment of *P. aeruginosa* infections (Vattem et al., 2007).

In this chapter, guanosine was selected as one of the potential QSIs compounds identified from the *in-silico* studies and evaluated further by targeting some virulence factors of pyocyanin production and motility to identify potential antivirulent agents of *P. aeruginosa*. The bioactive *M. comosus* extracts were subsequently added for evaluation to validate and compare whether the QSI was potent as a compound versus the mixture of compounds in relative extracts.

6.2. MATERIALS AND METHODS

6.2.1. Assessment of antiquorum sensing activities of compounds

Guanosine was the best compound tested further, this was done to validate the *in-silico* findings against *C. violaceum*, thus quorum sensing dependant violacein inhibition was performed. Qualitative and quantitative evaluation of the compound for antiquorum sensing activities was performed as described in section 2.2.5 with slight modifications.

6.2.2. Inhibition of quorum sensing mediated virulence determinants – pyocyanin assay

The pyocyanin method was performed to evaluate the inhibitory effects of *M. comosus* extracts (aqueous, methanol and dichloromethane) and guanosine to reduce the production of pyocyanin, as described by Bhattacharya et al. (2020), with slight modifications. Briefly, an overnight culture of *P. aeruginosa* in King's A broth was diluted to OD_{600nm} of 0.1. Subsequently, plant extracts and compounds were added with different concentrations (1.56 - 0.19 mg/mL: 2 x MIC - ¼ MIC), including the standardized culture and incubated overnight at 37 °C. A volume of 1.5 mL of overnight culture was centrifuged at 3000 rpm for 10 min. Afterwards, 1 mL of supernatant was transferred into fresh centrifuge tubes (pre-cooled in ice), allowed to chill and 100 µL chloroform was added while in ice. Then, 300 µL of 0.2 M hydrochloric acid (HCl) was added and mixed vigorously using a vortex mixer (EINS Sci E-VM-A, Biotechnology Hub Africa, Hatfield, South Africa). The chloroform layer containing pyocyanin was collected and transferred into a 96-wells microtiter plate. The absorbance was read at 520 nm using a microtiter plate reader (SpectraMax® paradigm). The experiments were performed in triplicates to get the mean value. Ciprofloxacin (0.001 mg/mL) and 1% DMSO were used as the positive and negative controls, respectively. Pyocyanin concentration was calculated by multiplying the OD value at 520 nm with 17.072 (the molar extinction coefficient). Pyocyanin production was compared with untreated cells and used as a control.

6.2.3. Swimming and swarming motility assay

The motility assay was performed following the method described by Yang et al. (2018), with slight modifications. Swimming media consisted of 1% tryptone, 0.5% NaCl, and 0.5% agar. The medium prepared for the swarming assay was composed of nutrient broth (0.8%, w/v), supplemented with glucose (3%, w/v) and agar of 0.5% (w/v). The standardized bacterium (2 μ L) was spotted on agar plates containing swimming and swarming media supplemented with or without extract. Each plant extract was tested at varying concentrations (2 x MIC - $\frac{1}{4}$ MIC in mg/mL) as well as guanosine (2 x MIC - $\frac{1}{4}$ MIC in mg/mL). Ciprofloxacin and 1% dimethyl sulfoxide were used as positive and negative controls, respectively. The plates were incubated at 37 °C for 24 h. The zone diameters (mm) were measured to assess swimming and swarming motility and compared to the negative and positive controls. The experiments were performed in triplicates, to obtain the mean value.

6.3. RESULTS

6.3.1. Qualitative and quantitative anti-quorum sensing activity of compounds

The violacein inhibition of *Melanthus comosus* extracts has already been reported in sections 2.3.3 and 2.3.4. Guanosine showed no inhibitory activity for the qualitative QS-dependent violacein inhibition, however quantitatively it showed varying inhibitory effects at MIC, $\frac{1}{2}$ MIC, $\frac{1}{4}$ MIC and $\frac{1}{8}$ MIC with 40.24, 33.61, 27.19 and 26.00 percentage inhibitory concentrations, respectively. The inhibitory concentration value (IC₅₀) was at 0.064 mg/mL, indicating at least some degree of activity.

6.3.2. Inhibitory effect of plant extracts and compounds on pyocyanin production

The three extracts (aqueous, methanol and dichloromethane) at different concentrations (2 x MIC, MIC, $\frac{1}{2}$ MIC and $\frac{1}{4}$ MIC) showed varying pyocyanin inhibitory activity. The highest concentration of 1.56 mg/mL (2 x MIC) showed the strongest inhibition on the production of pyocyanin (OD value: 2.04) for

dichloromethane extract and the same OD value of 2.22 for aqueous and methanol extracts while the lowest concentration 0.195 mg/mL ($\frac{1}{4}$ MIC) showed the weakest pyocyanin inhibition for aqueous and methanolic extracts at OD values of 3.41 and 3.24, respectively. Methanol extract however showed the least inhibition at $\frac{1}{2}$ MIC (OD value: 2.56) (Figure 6.1). Guanosine showed the highest pyocyanin inhibition at both 2 x MIC (0.062 mg/mL) and MIC (0.031 mg/mL) at an OD value of 2.39 while the least inhibition was at $\frac{1}{2}$ MIC and $\frac{1}{4}$ MIC (OD value: 2.73). The positive control (ciprofloxacin) showed pyocyanin inhibition at an OD value of 2.04 while the untreated cell showed lesser pyocyanin production at the value of 2.90 (Figure 6.1).

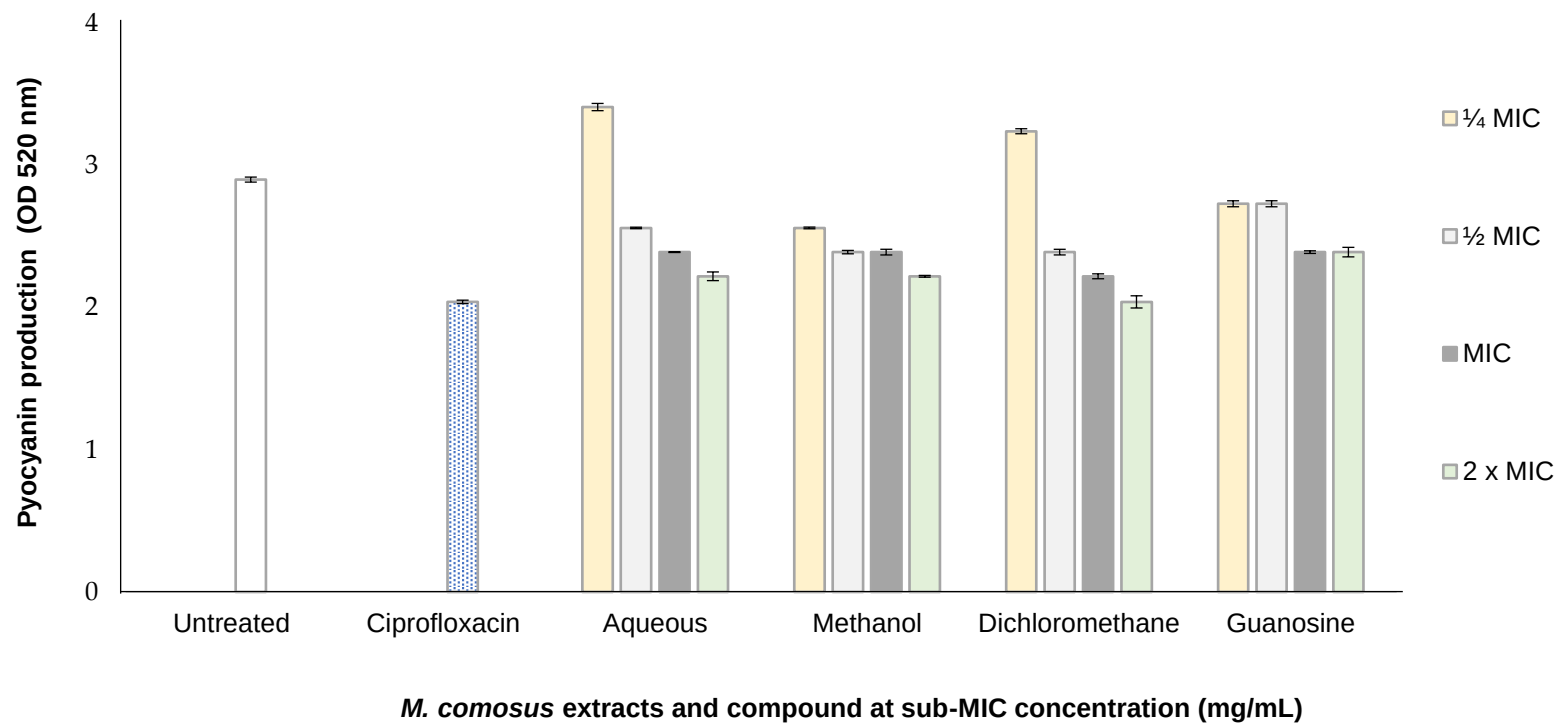


Figure 6.1: Pyocyanin production inhibition using *Melanthus comosus* (aqueous, methanol and dichloromethane) extracts and guanosine against *Pseudomonas aeruginosa* at a concentration between 1.56 and 0.195 mg/mL. Data are represented as optical density (OD) values read at an absorbance of 520 nm. Mean are values of triplicate independent experiments $\pm$ standard deviations. Untreated was set as a control for *Pseudomonas aeruginosa* pyocyanin production.

6.3.3. Effect of plant extracts and compounds on swimming and swarming motility

The swimming motility restriction was mostly observed at the highest concentration of plant extracts and guanosine as presented in Figure 6.2. In contrast to untreated cells (10 mm), the aqueous extract showed only a small restriction of motility at 2 x MIC with a 9 mm zone diameter, while the methanol extract only decreased motility at MIC (9 mm) concentrations. As shown in Figure 6.2, dichloromethane extract of *M. comosus* decreased swimming motility at all concentrations measured. Guanosine, on the other hand, inhibited swimming motility at 2 x MIC and MIC concentrations, with swimming zone diameters of 7 and 8 mm, respectively, as compared to the untreated cell, which had a swimming zone diameter of 10 mm. The three extracts and guanosine revealed swimming motility zones that can be compared with ciprofloxacin (positive control) showing a zone diameter of 9 mm (Figure 6.2).

All extracts of *M. comosus* at the four tested concentrations showed reduced swarming motility, although at varying diameter zones when compared to the untreated cells (14 mm, Figure 6.3). The highest level of inhibition was recorded at 2 x MIC concentration for all extracts and guanosine (Figure 6.3). Overall, guanosine revealed the best swarming motility restrictions, particularly at 2 x MIC concentration (8 mm zone diameter) which is comparable with ciprofloxacin, the positive control that showed significant swarming motility limitation (7 mm zone diameter).

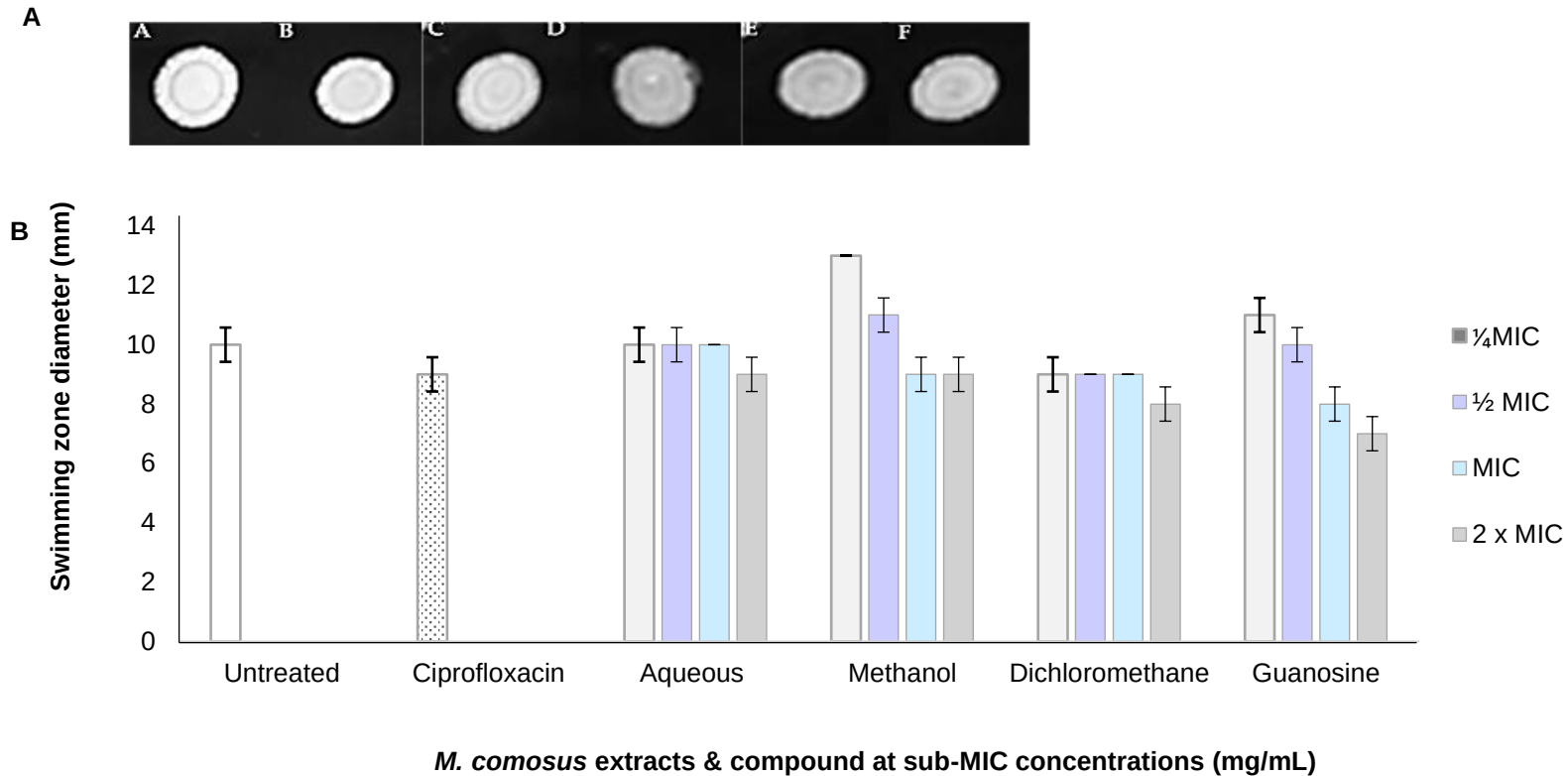


Figure 6.2A: (A) Negative control (untreated cells); (B) ciprofloxacin, (C) *Melianthus comosus* (aqueous), (D) *Melianthus comosus* (methanol), (E) *Melianthus comosus* (dichloromethane) extracts, respectively, and (F) guanosine are representative images of *Pseudomonas aeruginosa* swimming motility treated with *Melianthus comosus* (aqueous, methanol and dichloromethane) extracts and guanosine at 1.56 and 0.062 mg/mL (2 x MIC) concentration, respectively.

6.2B: Bar graph showing *M. comosus* extracts and guanosine at sub-minimum inhibitory concentrations. Mean values are of triplicate zone diameters.

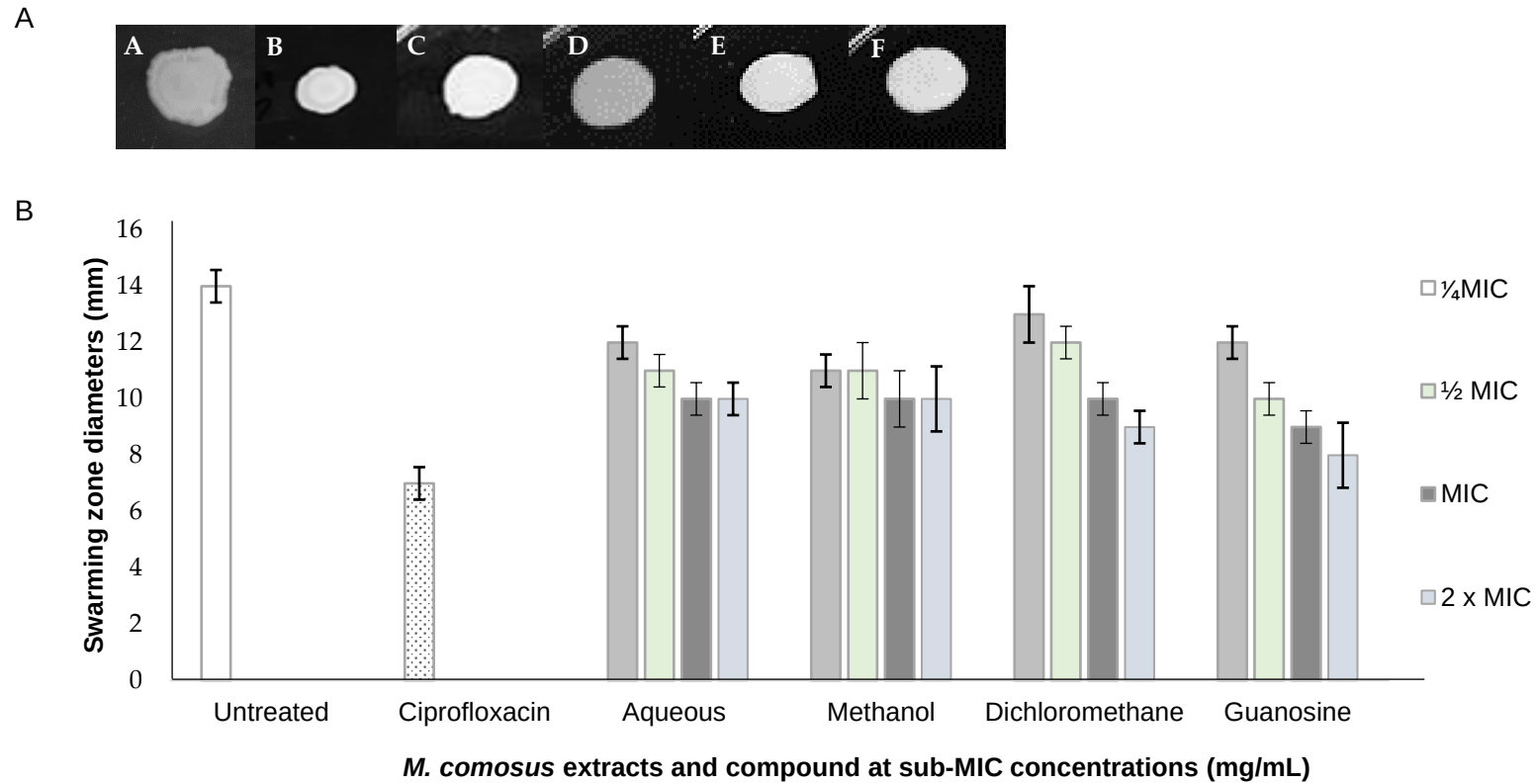


Figure 6.3A: (A) Negative control (untreated cells), (B) ciprofloxacin, (C) *Melianthus comosus* (aqueous), (D) *Melianthus comosus* (methanol), (E) *Melianthus comosus* (dichloromethane) extracts and (F) guanosine are representative images of *Pseudomonas aeruginosa* swarming motility treated with *Melianthus comosus* (aqueous, methanol and dichloromethane) extracts and guanosine at 1.56 and 0.062 mg/mL (2 x MIC) concentration, respectively, and **6.3B:** Bar graph showing *Melianthus comosus* extracts and guanosine at sub-minimum inhibitory concentrations. Mean values are of triplicate zone diameters.

6.4. DISCUSSION

The findings of this study indicate that guanosine, a plant-derived compound, is capable of inhibiting QS development, which is one of the reasons why plants have been used in traditional medicine. The quantitative AQS results of guanosine revealed as high as 40.24% of violacein inhibition at 0.75 mg/mL, which is quite comparable with the result of quercetin, the positive control that had 52.54 as its highest percentage of violacein inhibition at 0.75 mg/mL. This may be due to a similarity in the structure of guanosine with the AHL molecule, enabling structural analogues of signaling molecules (AHL) to competitively bind with corresponding receptor protein, influencing the transmission of signal molecules and quorum sensing. According to the current findings, guanosine, a purine nucleoside composed of guanine attached to a ribose (ribofuranose) ring through a beta N9 glycosidic bond, is a promising compound with the potential to inhibit biofilm formation and QS activity. Guanosine seems to be interesting as well because it is a purine nucleoside thought to have neuroprotective properties (Bettio, Gil-Mohapel & Rodrigues, 2016). Furthermore, when guanosine is phosphorylated, it can be converted into cyclic guanosine monophosphate, a secondary messenger that is needed for many physiological processes including visual transduction, gene expression, and metabolic function. On the other hand, quercetin is known for its pharmacological effects, inhibition of biofilm and ultimately, inhibition of quorum sensing as previously described (Thi, Wibowo & Rehm, 2020).

This study envisaged that plant extracts could prevent the formation of biofilm and therefore considered the alternative approach of inhibiting some extracellular factors in *P. aeruginosa* such as bacterial motility (swimming and swarming) and pyocyanin production. Pyocyanin, a blue secondary metabolite capable of producing free radicals, is one of the main virulence determinants of *P. aeruginosa*. This compound acts by interfering with ion transfer and mucus secretion in respiratory epithelial cells, which is usually seen in cystic fibrosis patients (Chong et al., 2018). Pyocyanin synthesis is often controlled by complex synchrony of QSS including the LasR-LasI, RhlR-RhlI, and PQS systems, which affect the development of rhamnolipids, proteases, and elastases (Lau et al., 2004). Results of the pyocyanin assay revealed varying inhibitory activity of the three extracts at different concentrations. The strongest pyocyanin inhibition was observed at the

highest concentrations of the aqueous, methanol and dichloromethane extract which suggests a correlation between concentration and the extent of inhibition. The degree of pyocyanin inhibition by extracts and guanosine can be compared to the positive control with a slight difference with an OD value of 0.18 against the untreated bacterium. In a study carried out by Al-haidari et al. (2016), pyocyanin level in *P. aeruginosa* treated with five plant extracts was also significantly reduced in contrast to the green pigment of untreated cultures. This could be explained as quorum control of pyocyanin production. Furthermore, quorum quenching agents have a significant effect on the release of pyocyanin by *P. aeruginosa* (Al-haidari et al., 2016).

Another reason for *P. aeruginosa* to be regarded as a major life-threatening opportunistic pathogen is its ability to colonize other environments through motility (Chong et al., 2018). QS regulates motility in *P. aeruginosa*, including swimming on a soft surface and swarming on a semisolid surface, which is facilitated by flagella and pili IV (Murray, Ledizet & Kazmierczak, 2010). The effects of *M. comosus* extracts and guanosine on the motility of *P. aeruginosa* investigated in this study exhibited a distinct influence on swimming and swarming motility compared to the negative control (untreated bacterial cells) as presented in Figure 6.2 and Figure 6.3, respectively. Plant extracts and guanosine treatment revealed a slight reduction in the swimming and swarming motility zones which corroborates the findings of Cosa et al. (2020) where *Calpurnia aurea* extracts reduced swimming and swarming motility in *P. aeruginosa*. Additionally, in a study carried out by Lakshmanan, Nanda & Jeevaratnam, (2018), the methanol extracts of *A. officinarum* and *C. tamala* significantly inhibited the swarming motility of *P. aeruginosa* when compared to untreated control. The swarming motility results were better than the swimming results obtained in the current study which is also in tandem with the submission of Lakshmanan, Nanda & Jeevaratnam, (2018). A reduction in the swarming area as compared to the control may indicate the presence of QSI compounds (Koh & Tham, 2011).

6.5. CONCLUSION

Guanosine tentatively identified in *M. comosus* extract has notably demonstrated to be responsible for the antivirulence activities against *P. aeruginosa*. *M. comosus* extracts and guanosine were capable of reducing the swimming and swarming motilities which form a significant part of the initial attachment of *P. aeruginosa* in causing an infection, while pyocyanin production is involved in evasion within the host. These promising findings show that guanosine may improve the host immune response and block the initiation of infection better than antibiotics with the potential of MDR.

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CHAPTER 7

GENERAL CONCLUSION AND RECOMMENDATIONS

7.1. General conclusions

- The suitable medicinal plants that could have the potential to manage multi-drug resistant bacteria were identified and validated in their antibacterial activities.
- Six bioactive plant extracts of *M. comosus* (aqueous, methanol and dichloromethane), *P. sidoides* (aqueous) and *V. karroo* (aqueous and methanol) were able to demonstrate antibacterial activities to potentially combat MDR bacteria and reduce the violacein production from the biomonitor strain *Chromobacterium violaceum*, thus chemical profiling was performed to ascertain which phytochemicals are responsible for their activity.
- Since biofilm formation of bacteria plays a significant role in MDR infections, it necessitates sourcing of novel alternative treatments. Here, selected bioactive plants and their phytochemicals proved to be able to hinder the cell attachment of Gram-positive bacteria (*Staphylococcus aureus* and *Streptococcus pyogenes*) although weaker inhibition was observed with Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*). It was rather difficult to eradicate an established biofilm.
- Through the use of *in-silico* studies, *C. violaceum* was the best screening model organism to identify potential phytochemicals with their quorum sensing inhibition mechanism of action.
- *In-silico* analysis performed on a model Gram-negative (MDR) pathogen of *P. aeruginosa* revealed guanosine as a potent compound that can be used to attenuate associated virulence factors of *P. aeruginosa*.
- The *in vitro* validation of *in-silico* findings proved guanosine to disarm *P. aeruginosa* QSS, reducing its virulence factors corroborating the *in-silico* analysis. This suggests that guanosine could potentially be used to manage *P. aeruginosa* infections and could potentially be tested on other GNB since it demonstrated positive findings in *C. violaceum*.

Overall, this study indicated that targeting the QSS of bacterial pathogens is a promising alternative to finding novel compounds that can be used to curb the ever-evolving MDR bacteria. The application of molecular docking and simulations highlighted the novel compound's ability to activate an inactive regulatory protein of *C. violaceum* and *P. aeruginosa*. Thus, suggesting suitable compounds that can be used as potential quorum sensing inhibitors.

7.2. Recommendations and limitations

This current study's findings presented the potential for quorum sensing inhibition to be used as an alternative strategy for the management of MDR bacteria. However, these studies should involve but not be restricted to the following:

- Evaluate the potential QS compounds of guanosine, phytol, 1,2,3-benzenetriol and α -D-glucopyranoside, methyl on other Gram-negative bacteria QSS, *in silico* and *in vitro*.
- Determine initial safety test of plant extracts and compounds using different cell lines suitable to be used on human hosts.
- *In-vitro* studies to validate the *in-silico* drug-likeness findings of the selected compounds before the *in-vivo* studies as this will reduce the time and costs.
- *In-vivo* studies using animal models to be performed on the potential antivirulent compounds to evaluate the side effects and safety of the compound.
- Determine the transcriptome measurements to confirm impact on gene expression
- The main limitation of using phytochemicals in antivirulence treatment could be insolubility in water and low bioavailability. However, these factors can be determined for human use by producing appropriate delivery methods for reaching a high therapeutic index.
- Another limitation is the planning of preclinical studies that considers only the anti-virulence properties as suitable without further *in-vivo* investigations.
- The efficacy and safety requirements of the potential antivirulence agents can be challenging.

APPENDICES

Appendix 1: Ethics approval for the use of multidrug-resistant (MDR) bacteria from the Faculty of Natural and Agricultural Science



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Natural and Agricultural Sciences
Ethics Committee
E-mail: ethics.nas@up.ac.za

27 July 2021

ETHICS SUBMISSION: LETTER OF APPROVAL

Miss IT Baloyi
Department of Microbiology and Plant Pathology
Faculty of Natural and Agricultural Science
University of Pretoria

Reference number: NAS158/2021

Project title: Anti-pathogenic properties of medicinal plants to combat carbapenem resistant Enterobacteriaceae infections

Dear Miss IT Baloyi,

We are pleased to inform you that your submission conforms to the requirements of the Faculty of Natural and Agricultural Sciences Research Ethics Committee.

Please note the following about your ethics approval:

- Please use your reference number (NAS158/2021) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
- Please note that ethical approval is granted for the duration of the research (e.g. Honours studies: 1 year, Masters studies: two years, and PhD studies: three years) and should be extended when the approval period lapses.
- The digital archiving of data is a requirement of the University of Pretoria. The data should be accessible in the event of an enquiry or further analysis of the data.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.
- **Applications using GM permits:** If the GM permit expires before the end of the study, please make an amendment to the application with the new GM permit before the old one expires
- **Applications using Animals:** NAS ethics recommendation does not imply that Animal Ethics Committee (AEC) approval is granted. The application has been pre-screened and recommended for review by the AEC. Research may not proceed until AEC approval is granted.

Post approval submissions including application for ethics extension and amendments to the approved application should be submitted online via the Ethics work centre.

We wish you the best with your research.

Yours sincerely,



Prof VJ Maharaj
Chairperson: NAS Ethics Committee