Evaluation of *Catha edulis* and *Thymus serrulatus* Extracts for their Antibacterial, Antioxidant, Antibiofilm and anti-Quorum-Sensing Activities on Selected Pathogenic Bacterial Species.

Yonatan Nesru Temam

A Thesis Submitted to the Department of Applied Biology

School of Applied Natural Science

Presented in Partial Fulfilment of the Requirement for the Degree of Master's in Biotechnology

Office of Graduate Studies

Adama Science and Technology University

June, 2023

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DECLARATION

I hereby declare that this Master's Thesis entitled "**Evaluation of *Catha edulis* and *Thymus serrulatus* Extracts for their Antibacterial, Antioxidant, Antibiofilm and anti-Quorum-Sensing Activities on Selected Pathogenic Bacterial Species.**" is my original work. That is, it has not been submitted for the award of any academic degree, diploma, or certificate in any other university. All sources of materials that are used for this thesis have been duly acknowledged through citation.

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Name of student	Signature	Date

RECOMMENDATION

We, the advisors of this thesis, hereby certify that we have read the revised version of the thesis entitled "**Evaluation of *Catha edulis* and *Thymus serrulatus* Extracts for their Antibacterial, Antioxidant, Antibiofilm and anti-Quorum-Sensing Activities on Selected Pathogenic Bacterial Species.**" prepared under our guidance by Yonatan Nesru submitted in partial fulfillment of the requirements for the degree of Masters of Science in Applied Biology. Therefore, we recommend the submission of the revised version of the thesis to the department following the applicable procedures.

Major advisor	Signature	Date
Co-advisor	Signature	Date

APPROVAL OF ADVISORS

We, the advisors of the thesis entitled "**Evaluation of *Catha edulis* and *Thymus serrulatus* Extracts for their Antibacterial, Antioxidant, Antibiofilm and anti-Quorum-Sensing Activities on Selected Pathogenic Bacterial Species.**" and developed by Yonatan Nesru, hereby certify that the recommendation and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

_____	_____	_____
Major advisor	Signature	Date
_____	_____	_____
Co-advisor	Signature	Date

APPROVAL OF BOARD OF EXAMINERS

We, the undersigned, members of the Board of Examiners of the thesis by Yonatan Nesru have read and evaluated the thesis entitled "**Evaluation of *Catha edulis* and *Thymus serrulatus* Extracts for their Antibacterial, Antioxidant, Antibiofilm and anti-Quorum-Sensing Activities on Selected Pathogenic Bacterial Species.**" and examined the candidate during the open defense. This is, therefore, to certify that the thesis is accepted for partial fulfillment of the requirement of the degree of Masters of Science in Biotechnology.

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Final, approval and acceptance of the thesis is contingent upon the submission of its final copy to the Office of Postgraduate Studies (OPGS) through the Department Graduate committee (DGC) and School Graduate Committee (SGC).

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School Dean	Signature	Date
Office of Postgraduate Studies, Dean	Signature	Date

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LIST OF ACRONYMS/ABBRIVATIONS

AHL - Acyl-homoserine lactone

AIP - Autoinducing peptide

AIs - Autoinducers

AMR - Antimicrobial resistance

CV - Crystal violet

C. edulis - Chata edulis

DCM - Dichloromethane

DMSO - Dimethyl sulfoxide

DPPH - 2,2-Diphenyl-1-picrylhydrazyl

EO - Essential Oil

EtAc - Ethyl Acetate

GC-MS - Gas Chromatography-Mass Spectrometry

LB - Luria Bertani

MBC - Minimum Bactericidal Concentration

MeOH - Methanol

MIC - Minimum Inhibitory Concentration

QS - Quorum Sensing

ROS - Reactive Oxygen Species

T. serrulatus - *Thymus serrulatus*

TPC - Total Phenolic Content

TFC - Total Flavonoid Content

WHO - World Health Organization

ABSTRACT

Antimicrobial resistance is progressively increasing at an alarming rate which make infections untreatable by conventional antibiotics over time. Natural products from plants are potential sources of possible solutions to combat this challenge. In this study, we have evaluated two plants, Catha edulis and Thymus serrulatus for phytochemicals, antibacterial, antioxidant, antibiofilm and anti-Quorum sensing potential. The GC-MS analysis of the Dichloromethane extracts identified phytol at 60.3%, Benzyl_carbazate at 20% and squalene at 4.4%; the ethyl acetate) extract contained (24R)-Stigmast-5-en-3.beta at 33%, β-Amyrin at 28%, squalene, α-Tocopheryl acetate at 4% and the essential oil had 1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)- at 35%, p-Cymene at 2% and Carvacrol methyl ether at 1%, respectively as the major compounds. Antibacterial activity was done by agar disk diffusion assay. All the extracts tested were able to inhibit bacterial growth: ethyl acetate (55-77%), Dichloromethane (53-73%), methanol (51-59) and essential oil (47-59%), respectively. Antioxidant activity was done by DPPH assay. The extracts exhibited high antioxidant property (78.3-89.18% radical scavenging activity). The minimum inhibitory concentration for the extracts of C. edulis were in the range (500-1000 µg/ml) and essential oil of T. serrulatus was 0.31-0.62 µl/ml, respectively. The minimum bactericidal concentration values were 2 mg/ml and 1.25-2.5 µl/ml for the same extracts. The Tolerance test values were in the bactericidal range of 2-4 for different bacteria except for P. aeruginosa that recorded a value of 8 and indicated bacteriostatic action. Antibiofilm activity was done by crystal violet staining assay. The methanol extract was found to have a dose-dependent inhibitory effect on biofilm formation in tested gram-positive bacteria, however, it did not exhibit a strong inhibition against biofilm formation in tested gram-negative that remained relatively constant at ~40% even at higher concentrations. The Dichloromethane extract demonstrated stronger inhibitory activity against gram-positive bacteria compared with gram-negative bacteria. The ethyl acetate extract and essential oil were able to effectively inhibit biofilm formation in gram-positive as well as gram-negative bacteria. All the C. edulis extracts exhibited inhibitory effects on pyocyanin production in P. aeruginosa (>44% at the highest concentration). The five major compounds generated during GC-MS analysis were evaluated for binding potential against four targets that are involved in Quorum sensing (LasI, LuxR, LsrR and AgrC) by molecular docking. All the targets bound the major phytocompounds with high energy and demonstrated strong binding indicating that these compounds may have the potential to effectively inhibit bacterial growth and biofilm formation via anti-Quorum sensing activity. Therefore, Catha edulis and Thymus serrulatus may be regarded as potential sources of antibacterial as well as precursors of individual antibacterial compounds with a potential to be investigated and developed further.

Keywords: *Catha edulis, Thymus serrulatus,* phytochemicals, Antimicrobial resistance, Antioxidant, Antibiofilm, Anti-quorum sensing, molecular docking

CHAPTER ONE

1. INTRODUCTION

1.1. Background and justification

Microorganisms evolve when external pressure is applied. Bacterial infections that once were easily treated by antibiotic use are now becoming untreatable and are a threat to global public health due to Antimicrobial resistance (AMR). Clinical resistance is a level of antimicrobial activity that is correlated with a high likelihood of therapeutic failure. In 2019, World Health Organization reported at least 700,000 deaths per year worldwide are caused by AMR and this could increase to up to 10 million deaths per year globally by 2050 if no action is taken (Organization, 2019). Therefore, scientists are looking for new ways to combat antibiotic-resistant bacteria by the development of therapeutics that target bacterial virulence mechanisms and virulence regulators, including those that control intercellular communication. The intra and inter-species communications could be antagonistic such as competition for nutrients or synergistic like biofilm development by co-aggregation, metabolic cooperation, and augmented resistance to antibiotics or host immune responses (Bhatt, 2018).

Quorum sensing (QS) is the regulation of gene expression regulated by chemical signal molecules called autoinducers (AIs) that are released depending on the change in cell population density (Miller & Bassler, 2001). The basic principles of QS remain unchanged in different bacteria although the signaling molecules used for QS can be very different. Two of the most studied systems are the acyl-homoserine lactone (AHL) QS systems in gram-negative and (cyclic) peptide QS systems in gram-positive bacteria (Schilcher & Horswill, 2020). When the concentration magnitude of the signal is above a certain threshold, the signal is detected by a QS receptor that elicits a downstream signal transduction cascade triggering a high cell density gene expression program including biofilm formation (Hawver, Jung, & Ng, 2016).

A biofilm is a collection of microorganisms whose cells adhere to a surface, creating a matrix of extracellular macromolecular substances composed of microorganisms and extracellular polymeric substances (EPS). EPS are organic polymers involved in bacterial interaction with their environment. EPS is mainly comprised of polysaccharides, proteins, extracellular DNA (eDNA), and lipids. The stability of the matrix is ensured by non-covalent bonding between EPS (Di Martino, 2018). Biofilms are known to be highly resistant not only to the host's

immune system but also to antibacterial agents withstanding 10 to 1000 times higher concentrations than cells in their plankton form. Antibiotic treatment can eliminate planktonic cells, however, it is difficult to target biofilms (Gilbert-Girard, Savijoki, Yli-Kauhaluoma, & Fallarero, 2020). Biofilm-related multi-drug resistance (MDR) has a high impact on hospital settings and the emergence of MDR. Biofilms are currently estimated to be responsible for more than 65% of nosocomial infections and 80% of all microbial infections (Jonnalagadda & Deshabathini, 2016; Magana *et al*., 2018). Microbial biofilms can lead to a wide variety of microbial infections in human health including Urinary Tract Infection, Catheter Infections, Middle Ear Infections, Plaque Formation, Gingivitis, and Contact Lens Plaque (Abraham *et al*., 2012). Predominant bacterial species that cause MDR include *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli, Staphylococcus epidermidis,* and *Viridans Streptococci. P. aeruginosa* has also been found to coexist with *S. aureus* in the lungs of cystic fibrosis patients and on wounds (Price *et al*., 2011; Willner *et al*., 2012).

In recent years, much of the research has been focused to identify alternative medicines to treat infections caused by drug-resistant organisms. The potential alternatives to chemical anti-biofilm agents are those of natural origin. Plant-derived molecules have found potential applications in the pharmaceutical industry. Plant extracts and other biologically active compounds isolated from leaves, stems, and roots have shown potential in their anti-biofilm activity. Natural extracts and natural product-based anti-biofilm agents are more efficient than the chemically synthesized counterparts with lesser side effects (Abraham *et al*., 2012; Mishra *et al*., 2020). Computational technique is used to predict and analyze the interactions between small molecules (ligands) and a target biomolecule (often a protein). It plays a crucial role in drug discovery and design, as well as in studying the potential of phytochemicals (plant-derived compounds) for various applications. (Azam & Abbasi, 2013).Targeting the attachment of bacterial cells and the development of biofilm structure is an ideal strategy for the inhibition of biofilm formation. In this study, potential medicinal plant (*Catha edulis* and *Thymus serrulatus*) extracts are studied for their anti-biofilm forming and anti QS abilities against clinically important bacterial isolates (*P. aeruginosa, E. coli, S. aureus,* and *S. pyogenes*).

1.2. Statement of the problem

Although equitable and affordable access to antimicrobials is still a problem in some parts of the world, the level of resistance to antimicrobial agents is becoming alarming in countries of all income levels, as common diseases are becoming more and more untreatable and medical procedures risker to perform. Biofilms pose a threat to antibiotic therapy due to their drug resistance. The economic damage of uncontrolled antimicrobial resistance would be devastating. Most of the studies that have been done in the past target inhibiting growth of pathogenic microorganisms with little effort on QS and antibiofilm inhibition. Understanding and identifying potential plants that inhibit biofilm-forming abilities by interfering with QS pathways of antimicrobial-resistant microorganisms is an alternative way to battle AMR.

1.3. Scope of the study

The study of microbial biofilm formation and QS signaling prevalent in antibiotic resistant bacteria is of paramount importance in a wide range of medical health and pharmaceuticals applications, and industries associated with health products and food industries. The study aims to contribute to the understanding of potential plants that can inhibit biofilm formation and interfere with the quorum sensing pathways of antibacterial-resistant microorganisms. The scope covers cell–cell communication and single cell interaction with the environment for the development of effective antibacterials for alternative strategies to combat antibacterial resistance and improve treatment options for infectious diseases.

1.4. Significance of the study

Commercial antibacterial agents are becoming less effective due to the emergence of resistant bacterial strains and biofilm-associated infections have become a major concern in healthcare settings, as they are notoriously difficult to treat due to their increased resistance to conventional antibiotics. Biofilms allow bacteria to evade the immune system and resist antibacterial agents. The emergence of antibiotic-resistant bacterial strains has further increased the challenge of combating biofilm infections One of the effective approach is to target bacterial cell-to-cell communication, QS.

Plant extracts have long been recognized for their diverse array of bioactive compounds, which possess antibacterial properties. Many medicinal plants of Ethiopia have been traditionally used for treating various diseases, and their therapeutic potential has been documented in traditional medicine systems. These plants offer a vast source of natural compounds that can potentially target biofilm formation and disrupt bacterial cell-to-cell communication, QS.

This study aimed to investigate the antibiofilm activity of *C. edulis* and *T. serrulatus* plant extracts against antibiotic-resistant bacteria known to form biofilms. By evaluating the efficacy of these extracts, we seek to identify novel natural compounds that can inhibit biofilm formation and potentially serve as a basis for the development of new antibacterial drugs.

Understanding the antibiofilm potential of medicinal plants not only expands the treatment options but also contributes to the sustainable utilization of natural resources. Furthermore, the exploration of traditional medicinal plants in the context of modern antibacterial therapy presents an opportunity to bridge traditional knowledge with contemporary scientific advancements, potentially unlocking new avenues for drug discovery.

The findings of this study will provide valuable insights into the potential of medicinal plants as a viable alternative to conventional antibacterial agents. This research may ultimately contribute to the development of novel therapeutic approaches to combat biofilm-associated infections and address the growing challenge of antibiotic resistance in healthcare settings.

1.5. Objectives of the study

1.5.1. General objective

To evaluate the potential of medicinal plants extracts for inhibiting biofilm formation and their ability to interfere with QS activities of antibiotic resistant bacterial species.

1.5.2. Specific objectives

Specific objectives of this study are to:

- Identify compounds present in extracts using qualitative screening and Gas Chromatography Mass Spectroscopy (GC-MS) analysis.
- Evaluate the antibacterial activities of extracts.
- Determine the minimum concentration of plant extracts that will inhibit bacterial growth (MIC) and the minimum bactericidal concentration of plant extracts that will completely kill bacterial cells (MBC).
- Stain and quantify biofilm formation by using UV spectrophotometry analysis.
- Quantify Pyocyanin molecules for the assessment of the anti QS ability of plant extracts in *P. aeruginosa*.
- Determine the interaction of compounds from *C. edulis* extracts with different anti QS targets using molecular docking simulation.

CHAPTER TWO

2. LITRATURE REVIEW

2.1. Antibiotic resistance

Antibiotic resistance is an escalating issue that arises when bacteria undergo changes in response to antibiotic usage. The development of antibiotic resistance occurs solely in bacteria, not in humans or animals. These resistant bacteria can infect both humans and animals, leading to infections that are more difficult to treat compared to those caused by non-resistant bacteria (Shrivastava, Shrivastava, & Ramasamy, 2018). Bacteria can acquire resistance to antibiotics through various mechanisms. These include altering the target site of the antibiotic, producing enzymes that render the antibiotic inactive, and decreasing the uptake of the antibiotic into bacterial cells (Yalew, 2020).

2.1.1. Biofilm-mediated antibiotic resistance mechanisms

One of the main properties of bacterial biofilms is their capacity to be more resistant to antibiotic agents which makes infections difficult and sometimes impossible to treat. Biofilm structures show maximum resistance to antibiotics in the mature stage (Srivastava & Bhargava, 2016). Biofilms confer less susceptibility to specific antibacterial agents by combination of different factors namely: (i) a poor antibiotic penetration into the polysaccharide matrix; (ii) the arbitrary presence of cells showing a resistant phenotype (known as “persisters”); and (iii) the presence of either non-growing cells or cells that triggered stress responses under unfavorable chemical conditions within the biofilm matrix (Balcázar, Subirats, & Borrego, 2015).

A. Gene transfer

Mutation is likely to occur in biofilm-growing bacteria compared with planktonic isogenic bacteria. Biofilms provide suitable environments for bacterial interactions because of cell-cell contact thus High population densities and proximity of cells in biofilms also increases the chances for genetic exchange among bacterial species converting biofilms in to hot spots of antibiotic resistance by horizontal gene transfer and concentration of communication signals and extra cellular DNA (Balcázar *et al*., 2015; Varadarajan *et al*., 2020). The exchange of genetic material through conjugation is one of the most efficient pathways to spread antibiotic resistance among bacterial cells. The efficiency of gene transfer seems to be correlated with the biofilm surface, suggesting that a high surface/volume ratios favor

transfer within or between biofilm populations. High plasmid transfer rates occur in biofilm populations independent of the nutrient concentration in the medium (Molin & Tolker-Nielsen, 2003). Bacterial conjugation is a common mechanism and the most problematic for horizontal gene transfer and the spread of antibiotic resistance among bacteria (Graf, Palm, Warringer, & Farewell, 2019). Relative spatial stability of bacterial biofilms favor conjugation. Conjugation is mainly mediated by conjugative plasmids but conjugative transposons are also capable of triggering the process. Conjugative plasmids can be exchanged either between related bacteria or phylogenetically different bacteria (Balcázar *et al*., 2015).

B. **β-lactamase production**

Beta-lactam antibiotics are one of the most commonly prescribed drug classes for the management and treatment of bacterial infections. They have a common feature which is the 3-carbon and 1- nitrogen ring (beta-lactam ring) that is highly reactive (Pandey & Cascella, 2019). The most common mechanism of acquired resistance to β-lactam antimicrobials is β-lactam destruction by β-lactamase enzymes (chromosome-encoded or expressed from acquired mobile genetic elements) (Poole, 2007). For instance, overproduction of the chromosomally-encoded AmpC β-lactamase (e.g cephalosporinase) is believed to be the primary mechanism of resistance of *P. aeruginosa* isolates to β-lactam antibiotics. The diffusion barrier of EPS contributes to AMR in β-lactamase overproduction and hydrolyzing β-lactam antibiotics before reaching the bacterial cells (Ciofu & Høiby, 2007).

C. **Efflux pumps**

Efflux pumps are widely involved in MDR because they can extrude majority of clinically important antibiotics from cells. Efflux mechanisms in both drug-specific and multidrug are important parts of naturally or acquired resistance. Multidrug efflux mechanisms are generally chromosomally encoded and their expression usually results from mutations in regulatory genes, whereas drug-specific efflux mechanisms are encoded by mobile genetic elements and their acquisition is sufficient for tolerance (Piddock, 2006). MDR efflux Pumps contribute to bacterial antibiotic resistance in several ways: (i) inherent resistance to entire drug classes, (ii) inherent resistance to specific drugs, and (iii) by overexpression of efflux pumps. Endurance granted (Piddock, 2006). Efflux pumps like MDR are also involved in autoinducers cross membrane trafficking in QS and are also directly involved in the

formation of biofilm. Studies show inhibition of efflux activities by efflux pump inhibitors (EPIs) reduce biofilm formation significantly (Sun, Deng, & Yan, 2014).

2.2. Biofilm formation process

Microorganisms mostly exist in the environment in the form of biofilms. Almost all bacteria (gram-positive or gram-negative, pathogenic or non-pathogenic) can form biofilms (J. Zhou & Cai, 2018). The developmental stages leading to biofilm formation appear to be conserved but, every species form a unique multicellular community. The biofilm formation process can generally be divided into four main phases (Figure 1): (i) initial attachment, (ii) division into microcolonies and production of extracellular matrix, (iii) maturation and cell proliferation, (iv) aging and dispersal (J. Zhou & Cai, 2018).

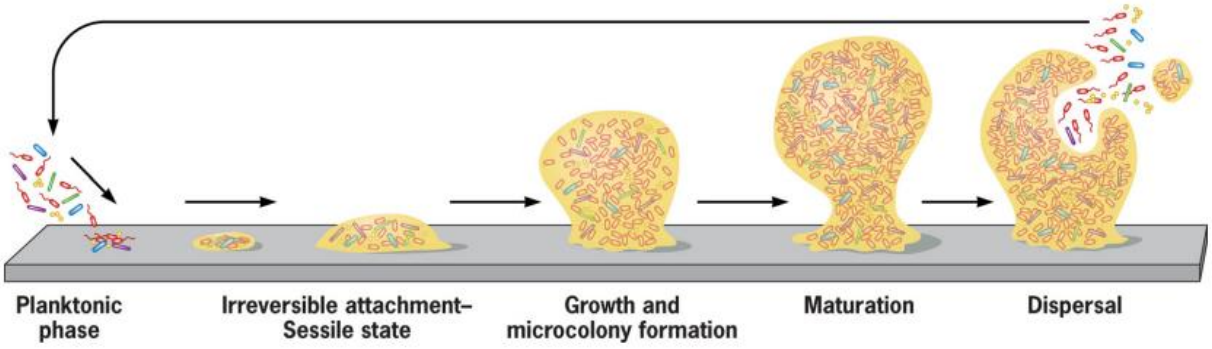

Figure 1. Developmental stages of biofilm formation. Planktonic bacterial species attach to biotic/abiotic surfaces. Adherent bacteria grow as multicellular communities and form microcolonies that proliferate and mature. This microbial infrastructure leads to the development of mature biofilms. Finally, biofilms act as bacteria "Reservoirs" that are brought back into the environment by biofilm diffusion and colonize new surfaces (adapted from (Magana *et al*., 2018)).

The formation of biofilm is mainly regulated by Quorum sensing (QS). The signaling molecules used for QS can be very different depending on the species (Schilcher & Horswill, 2020).

2.3. Mechanisms of biofilm formation regulated by QS

Based on the type of autoinducers (AIs) employed, QS mechanisms can be generally divided into AHL system and AIP system. The AHL system is present in gram-negative bacteria and the signaling molecule used in this system is N-acylhomoserine lactone (AHL). The AIP system uses an autoinducing peptide (AIP) and is found only in gram-positive bacteria. (Schilcher & Horswill, 2020).

2.4. AHL-Mediated QS biofilm formation in gram negative bacteria

In gram-negative bacteria (such as *P. aeruginosa*), the production of the small diffusible signal molecules AHLs involves LuxI synthase (Figure 2). When AHLs accumulate in the environment and reach a high concentration, AHLs bind with LuxR activator protein and synchronously induce lux-operon transcription. Many gram-negative bacteria use similar LuxI-type synthases and LuxR-type activator proteins (Deryabin *et al*., 2019; Hamde *et al*., 2022).

P. aeruginosa responds to AHLs and possesses four AHL quorum-sensing systems/pathways: Las, Rhl, PQS, and IQS (Thi, Wibowo, & Rehm, 2020). Las and PQS are triggered by an increased cell density at the preliminary exponential growth phase and each system has its own AHL synthase (LasI and RhlI) and its transcriptional regulator (LasR and RhlR) (Schaefer *et al*., 2013). The AHL signals produced by the synthases are *N*-(3-oxododecanoyl)-HSL and *N*-butyryl-HSL, respectively whereas PQS and IQS systems are activated at late exponential growth phase, especially under iron limitation. Additionally, the PQS system has been reported to be related to the synthesis of bacterial extracellular DNA, which is important for the formation of biofilms (López, Vlamakis, & Kolter, 2010; J. Zhou & Cai, 2018). The four systems AHL quorum-sensing systems/pathways: Las, Rhl, PQS, and IQS cross-interact to form a complicated QS network that co-regulates biofilm formation by *P. aeruginosa.*

LuxS and LsrR are two proteins involved in bacterial quorum sensing, which is a cell-to-cell communication mechanism commonly used by *E. coli*. LuxS is an enzyme that catalyzes the production of autoinducer-2 (AI-2) signaling molecule, which is a component of the methyl cycle and methionine metabolism. LsrR is a transcriptional repressor that binds to the promoter regions of genes involved in AI-2 uptake and metabolism which triggers an expression (Xue, Zhao, Sun, Zhou, & Sun, 2009).

Type IV pili (TFP) are important colonization factors of the opportunistic pathogen *P. aeruginosa* involved in biofilm formation, attachment to host cells, for twitching and motility. Bacterial cells without TFP have been shown to be significantly less able to bind to eukaryotic cell (Kus, Tullis, Cvitkovitch, & Burrows, 2004). The major subunit of TFP is a protein encoded by the *pil*A gene. Twitching motility is involved in biofilm architecture and is also responsible for the formation of microcolonies in biofilms. Swarming and swimming motilities are mediated by flagella and allow the movement of the microorganism on surfaces and in aqueous environments (Horna *et al*., 2019).

The *pil*A gene is found at a conserved chromosomal locus between the adjacent *pil*B and *t*ARN^{Thr} genes. In addition, *pil*A has five pili alleles with its accessory genes (*tfp*Oa, *tfp*Ob, *tfp*W, *tfp*X, *tfp*Y, *tfp*Z) (Kus *et al*., 2004).

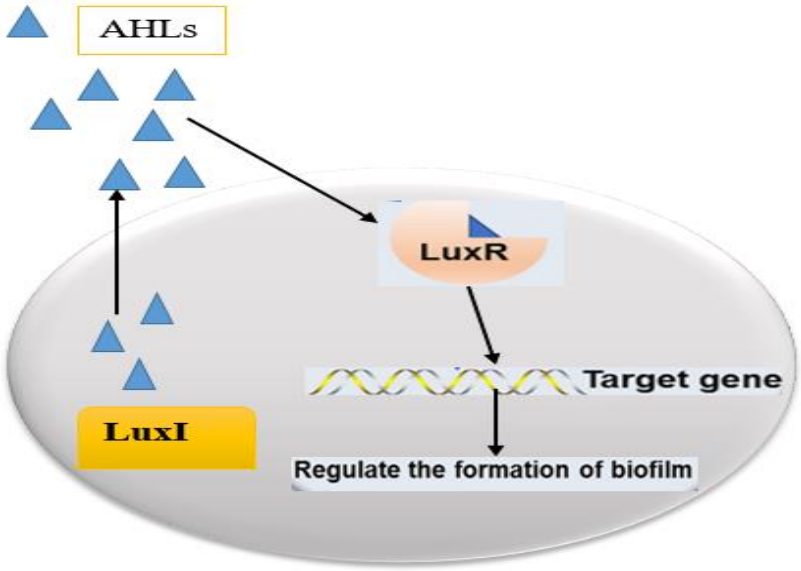

Figure 2. AHL mediated QS biofilm formation of gram-negative bacteria: the autoinducer AHL is synthesized by LuxI synthase and released outside, when extracellular AHL concentration reaches its threshold entering the cell it interacts with signal receptors and triggers target genes which are responsible for biofilm formation.

2.5. API mediated QS biofilm formation in gram-positive bacteria

Autoinducing peptide (AIP)-mediated regulation of biofilm formation by the QS system occurs when bacteria produce small oligopeptides intracellularly, which are processed into mature AIPs by modification and transported out of the cell transmembrane protein, is responsible for the conversion of AgrD to mature AIP and transportation of the resulting AIP outside of the cell (Figure 3). When AIP reaches a threshold concentration, it binds to the extracellular segment of histidine kinase, a transmembrane receptor present in the cell membrane, activating the kinase, phosphorylating downstream response regulators, and regulating expression of genes associated with biofilm formation (L. Zhou, Zhang, Ge, Zhu, & Pan, 2020).

In *S. aureus* five genes (*agr*A, *agr*B, *agr*C, *agr*D and *hld*) in the *agr* operon are involved in the *agr* AIP system (Painter, Krishna, Wigneshweraraj, & Edwards, 2014).

The signal molecules for QS in gram-positive bacteria are peptides. Among the diversified structure peptides employed as QS signal molecules, the Agr-type autoinducing peptide (AIP) with a thiolactone/lactone structure has been found commonly used in *S. aureus* (L. Zhou *et al*., 2020).

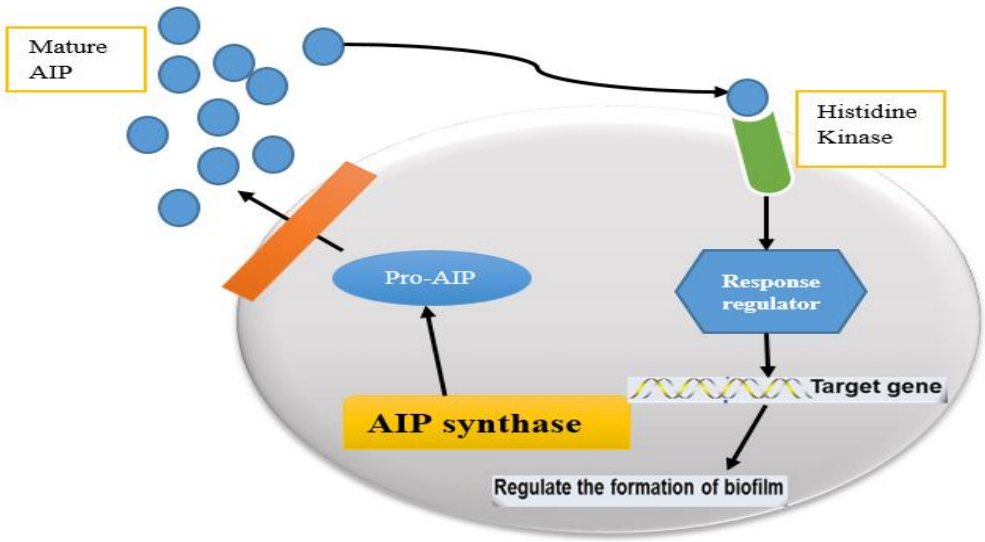

Figure 3. API mediated QS biofilm formation of gram-positive bacteria: the pro-AIP synthesized by AIP synthase and converted to mature AIP and transported outside the cell by transmembrane protein. When extracellular AIP concentration reaches its threshold, AIP binds to histidine kinase receptor activating the kinase, phosphorylating downstream response regulators, and regulating expression of genes associated with biofilm formation.

In methicillin resistant *Staphylococcus aureus* (MRSA) the expression levels of biofilm related genes encoding fibronectin binding protein A and B and clumping factor B (*fnb*A/B and *clf*B) which are involved in the primary adhesion of *S. aureus* are significantly increased at 24h and slightly decreased at 48 h. The RNA expression of elastin binding protein (*ebps*) is significantly increased (6×) at 24h compared to 12 h (Atshan *et al*., 2013). The *ica*ADBC operon are responsible for the production of necessary proteins for the synthesis of polysaccharide intracellular adhesion (PIA). Other genes biofilm related genes are summarized in table 1.

Table 1. Some other biofilm related genes and their functions

Gene(s)	**Function**	**References**
psl gene c (pslA to -F)	Involved in biofilm formation, protection against aminoglycoside antibiotics, and increased the elasticity and effective cross-linking within the matrix, which strengthened its scaffold and appeared to facilitate the formation of microcolonies.	(Chew *et al*., 2014; Jackson, Starkey, Kremer, Parsek, & Wozniak, 2004)
pel genes	Seven genes involved in pellicle matrix formation, and required for close association of species in mixed-species microcolonies (e.g. *P. aeruginosa and s. aureus*)	(Chew *et al*., 2014; Magana *et al*., 2018)
QS genes (lasIR, rhlIR)	Cell-to-cell communication enhancement, coding for AHL synthase, biofilm formation, resistance, motility	(Cabrol, Olliver, Pier, Andremont, & Ruimy, 2003; Hemati *et al*., 2014; J. Zhou & Cai, 2018)

2.6. Strategies for the inhibition of biofilm formation

An ideal strategy for the inhibition of biofilm formation is targeting the attachment of bacterial cells and development of biofilm structure. QS plays a key role in the formation of natural biofilm agents. There are various natural anti-biofilm agents including phytochemicals, bio-surfactants, antimicrobial peptides, and microbial enzymes that have different mechanisms of action i.e. via interfering the quorum-sensing pathways (Figure 4) (Mishra *et al*., 2020).

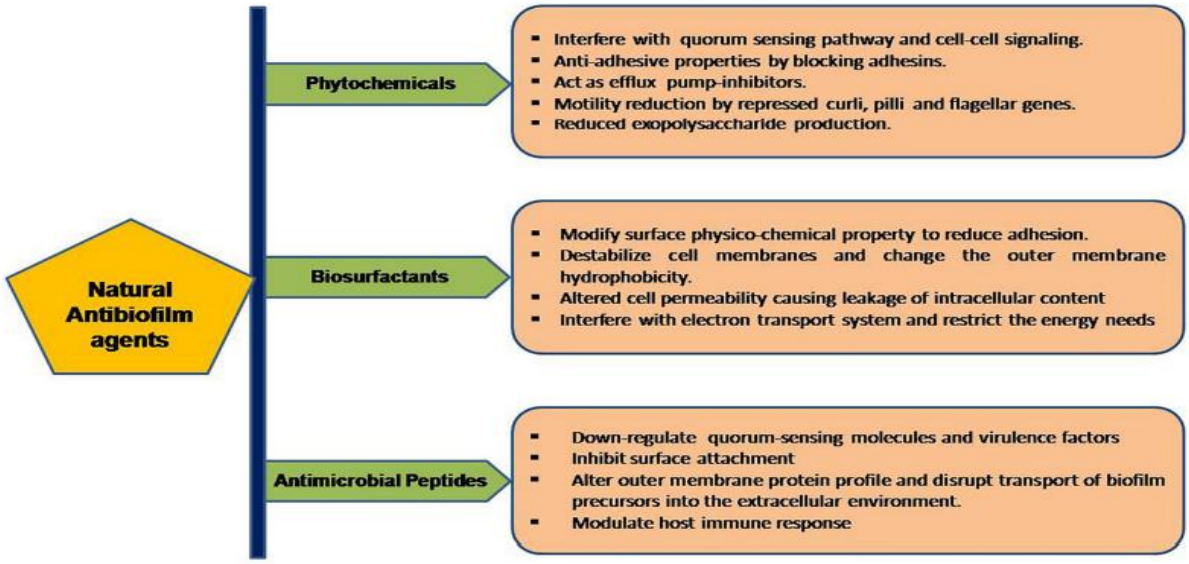

Figure 4. Flowchart review of natural antibiofilm agents adapted from (Mishra *et al*., 2020).

2.7. The role of medicinal plant extracts in combating antibiotic resistance, biofilm formation for anti-quorum sensing.

Plant extracts and other biologically active compounds isolated from leaves, stems, and roots have shown potential in their anti-biofilm activity. Plant extracts containing antioxidants can be beneficial for human health. Antioxidants can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. Studies have shown that plant polyphenols can be used as antioxidants against different oxidative stress-induced diseases. Natural extracts and natural product-based agents are more efficient than the chemically synthesized counterparts with lesser side effects (Abraham *et al*., 2012; Mishra *et al*., 2020).

2.7.1. Phytochemicals

Natural compounds obtained from plants with potent anti-biofilm properties are broadly classified into phenolics, essential oils, terpenoids, lectins, alkaloids, polypeptides, and polyacetylenes. Phenolics are a group of compounds consisting of seven subclasses including phenolic acids, quinones, flavonoids, flavones, flavonols, tannins and coumarins (Yong, Dykes, & Choo, 2019).

Phytochemicals inhibit the quorum sensing mechanism mainly by blocking the quorum sensing inducers like AHL and autoinducers. They also play a significant role in inhibiting bacterial adhesions and suppression of genes related to biofilm formation (Mishra *et al*., 2020).

2.7.2. Inhibition of QS in bacteria

Flavonoids from plants are known for their pharmacological effects such as reducing the production of pyocyanin and inhibiting the biofilm formation in *P. aeruginosa.* According to (Ouyang *et al*., 2016), flavonoids significantly inhibit the biofilm formation and production of virulence factors including pyocyanin, protease, and elastase at a lower concentration than those for most previously reported plant extracts and substances. It also significantly reduces the expression levels of LasI, LasR, RhlI, and RhlR without impacting the growth of *P. aeruginosa.* Administration of the flavonoids to *P. aeruginosa* alters transcription of quorum sensing-controlled target promoters and suppresses virulence factor production, confirming their potential as anti-infectives that do not function by traditional bactericidal or bacteriostatic mechanisms (Paczkowski *et al*., 2017; Thi *et al*., 2020).

A high amount of Flavonoid glycosides (e.g quercetin) are found in the plant *C. edulis* (khat). These compounds are very effective in inhibiting the biofilm formation of *P. aeruginosa* by targeting the LasR and RhlR receptors (Ouyang *et al*., 2016; Paczkowski *et al*., 2017; Sagar, Aneesha, & Uppin, 2018). Tannic acid has been shown to be an effective biofilm formation inhibitor in *S. aureus* by directly binding to peptidoglycan of the cell wall (Dong *et al*., 2018). Carvacrol is known for its inhibition of QS-regulated violacein biosynthesis and pyocyanin production (Myszka *et al*., 2016).

2.8. *C. edulis* (khat)

C. edulis known as khat is an evergreen plant indigenous to Ethiopia and Yemen as the countries of origin and is also found in many other eastern and southern African countries, however, recently its cultivation spread further to Madagascar and as far as Afghanistan. Khat is mostly chewed by the local people for its stimulant action. The effects of khat consumption include euphoria, excitation, anorexia, increased respiration, hyperthermia, logorrhea, analgesia, and increased sensory stimulation (Feyissa & Kelly, 2008; Lemessa, 2001).

C. edulis consists of many compounds that belong to flavonoids, alkaloids, tannins, classes of phenylalkylamines and cathedulins, sterols and triterpenes, monoterpenes and volatile aromatic compounds, and other miscellaneous compounds like amino acids and vitamins in the leaves and roots (Getasetegn, 2016). Early attempts attributed the stimulating effects of khat to cathine, a phenylalkylamine-type constituent characterized as (+)-norpseudoephedrine. Proanthocyanidin technique was used to detect tannin acid and the result showed that khat contained high concentration of condensed tannin (25.4%) based on the chemical analysis of extracts. Every 1 g of khat extracts contained 254 mg of condensed tannins (Al-Alimi, 2017).

C. edulis is known for its calming effect thus it's known for its abusive usage. However, khat also provides some medicinal potential. In recent years, many medicinal values of Catha edulis have been discovered gradually. Callus of Catha edulis have "HIV-1" reverse transcriptase inhibition effects and exhibit high antibacterial properties against both gram-positive and gram-negative bacteria compared to plant leaves (Ye, Hu, Liu, & Liang, 2021). The use of khat for medicinal treatment is not yet fully understood, but it has been used to treat serious diseases such as gonorrhea, asthma, chest complications, depression, gastric ulcers, obesity, and tiredness (Abou-Elhamd *et al*., 2021).

2.9. *T. serrulatus* Hochst (Tosign)

In different parts of the world, various species of *Thymus* (*T. linearis, T. vulgaris,* and *T. serphyllum*, etc.) are known for their traditional uses in the treatment of respiratory disorders such as bronchitis, asthma, pertussis, laryngitis, tonsillitis, and cough caused by common colds. *T. serrulatus* is indigenous to Ethiopia and traditionally, it is extensively used for various disease conditions including flu and cough (Sintayehu, 2020). In various scientific studies, this plant has been found to be active against various bacterial strains. Additionally, previous studies have shown that the pharmacological activities of the essential oil are positively correlated with the presence of active ingredients such as thymol, carvacrol, p-cymene, γ-terpinene, and rosmarinic acid (N. U. Rehman *et al*., 2021).

T. serrulatus is used to cure various diseases and as a food ingredient. In the Ethiopian folk medicine, the decoction is orally taken as a remedy to treat diabetes and high blood pressure. The essential oil of *T. serrulatus* is also used for medicinal purposes as antiseptic, antifungal, and vermifuge properties (Haile *et al*., 2021).

T. serrulatus and *T. schimperi* are used as traditional medicine for the treatment of headache, cough, stomachache, earache, liver disease and gonorrhea. They are also used as flavor tea, coffee and different kinds of stew. In Ethiopia *T. schimperi* possesses antibacterial, anthelmintic and antifungal activity (Abera *et al*., 2019). Thymus serrulatus and Thymus schimperi belong to the chemotypes Carvacrol-Thymol. Carvacrol (63%), thymol (36%-38%), Thymol (49%) are the major constituent of the essential oil of T. schimperi collected from Bale, T. schimperi from Gonder, Shewa & Wello, T. serrulatus from the Tigray region respectively (Haile *et al*., 2021; Memar, Raei, Alizadeh, Aghdam, & Kafil, 2017).

2.10. Extraction of phytochemicals

Phytochemicals can be extracted from different plant parts using selective solvents by different methods such as maceration, soxhlet, hydrodistillation, sonication, and many more procedures based on the targeted phytochemicals and the desired level of purity (A. Altemimi, N. Lakhssassi, A. Baharlouei, D. G. Watson, & D. A. Lightfoot, 2017). Each extraction method has its own advantages and limitations, making it crucial to select the most suitable technique for the specific phytochemical of interest. Maceration is a very simple and efficient way of extraction which involves soaking the plant material in a solvent for an extended period of time to allow the compounds to dissolve gradually (Al Ubeed *et al*., 2022).

Methanol (MEoH) is considered as an effective solvent for plant extractions, given it is a highly polar solvent, its highest extraction yields, and highest content of phytochemicals like flavonoid, alkaloid, and terpenoids and methanol (Felhi *et al*., 2017; Idris & Mohd Nadzir, 2021).Ethyl acetate and dichloromethane are commonly used solvents for the extraction of phytochemicals from plant materials. Ethyl acetate offers advantages such as low toxicity, good selectivity for various phytochemicals, moderate polarity (Supang *et al*, 2022).

Dichloromethane provides excellent solvent power for a wide range of phytochemicals, especially non-polar and moderately polar compounds. Its low boiling point and high volatility enable rapid extraction, making it effective for extracting compounds (Cequier-Sánchez *et al*., 2008).

Hydrodistillation has long been used for the extraction of essential oils and bioactive compounds from plant materials. The Clevenger apparatus is used for the extraction of volatile oils (Park & Tak, 2016).

2.11. Molecular docking

Molecular docking is a computational technique used to predict and analyze the interactions between small molecules (ligands) and a target biomolecule (often a protein). It plays a crucial role in drug discovery and design, as well as in studying the potential of phytochemicals (plant-derived compounds) for various applications. (Azam & Abbasi, 2013).

2.11.1. Benefit of using CB-Dock protein-ligand docking method

CB-Dock employs a novel curvature-based cavity detection approach to predict binding sites on a given protein. This eliminates the need for users to provide preset binding sites, which can be time-consuming and require prior knowledge (Liu *et al*., 2020).

CB-Dock utilizes Autodock Vina, a widely used and popular docking program, for performing the docking calculations. This integration ensures compatibility and takes advantage of the robust docking algorithms offered by Autodock Vina (Morris & Corte, 2021).

CB-Dock has undergone careful optimization to improve its performance. It achieved a success rate of approximately 70% for top-ranking poses with root mean square deviation (RMSD) within 2 Å from the X-ray pose. This performance outperformed state-of-the-art blind docking tools in benchmark tests, indicating its effectiveness in predicting accurate binding modes (Liu *et al*., 2020).

CHAPTER THREE

3. MATERIALS AND METHODS

3.1. Sample collection

3.1.1. Bacterial sample collection

The microorganisms (*P. aeruginosa* ATTC27853, *E. coli* ATTC25922, *S. aureus* ATTC25923, and *S. pyogenes* ATTC12204) were obtained from laboratory stock of Adama Regional Laboratory, Adama and cultured aerobically in Luria-Bertani (LB) broth overnight at 37°C.

3.1.2. Plant sample collection

Plant samples were collected from different locations of Ethiopia (Hosanna for *C. edulis* and Galama mountain near Bekoji for *T. serrulatus*) based on suitable climate and abundance of plants (Figure 5).

Hosanna is a town and separate woreda in southern Ethiopia, and the administrative center of the Hadiya Zone. It is located in the Southern Nations, Nationalities, and People's Region (SNNPR), Hosanna has a latitude and longitude of 7°33′N 37°51′E with an elevation of 2,177 meters.

Bekoji is a town in central Ethiopia. It is located in the Arsi Zone of the Oromia Region and has a latitude and longitude of 7°35′N 39°10′E with an elevation of 2810 m. Galama is a mountain in Ethiopia with an elevation of 3,697 meters. It is located in the Arsi Mountains National Park which was designated in 2011 and covers an area of 10,876 km².

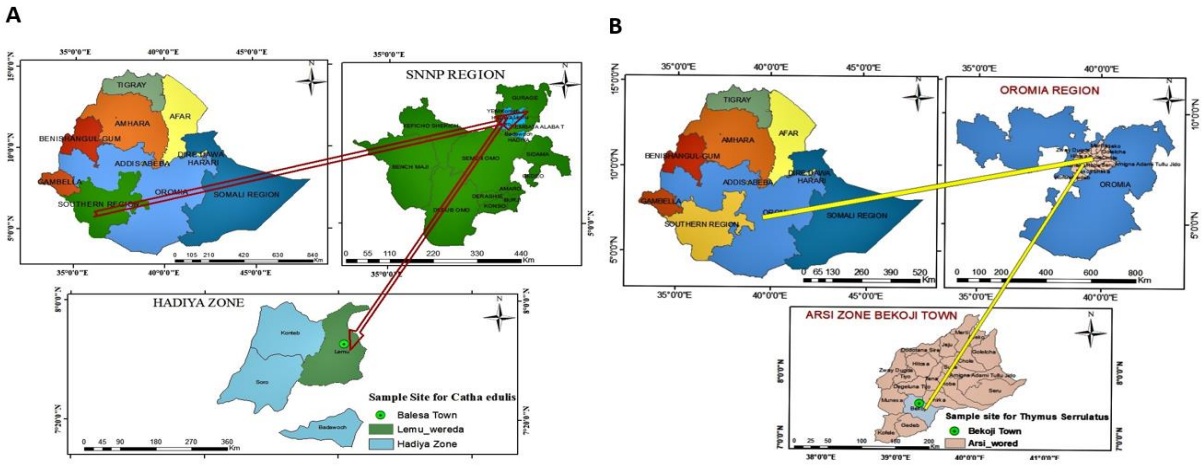

Figure 5. Global information system (GIS) for sample collection area of *C. edulis* (A) and *T. serrulatus* (B).

3.2. Preparation of plant extracts

A. C. edulis

Methanol extraction of *C. edulis* was performed by using maceration and ultrasonic assisted extraction (UAE) methods. The fresh leaves were rinsed with distilled water and allowed to dry at room temperature indoor without sunlight contact. The dried leaves were ground into a powder and a total of 300 grams of the powdered leaves was then mixed with 1.5 L of 99% methanol (1:5) and sonicated for 30 min and left to macerate with intermittent shaking at 120 rpm for three days. After the solution was filtered with Whatman filter paper No. 1, the filtrate was concentrated using a rotary evaporator at 40 °C.

The methanol extract was further extracted by liquid-liquid extraction process was used to obtain extracts of Dichloromethane (DCM), and Ethyl acetate (EtAc) as described by (Schmidt & Strube, 2000) with slight modifications. Briefly, in a separatory funnel MeOH crude extract was homogenized with methanol and equal volume of DCM after shaking, the mixture was then allowed to sit, enabling phase separation. This resulted in two distinct layers, each containing different compounds dissolved in their respective solvents. The bottom (MeOH) layer was carefully drained and a new batch of DCM was added into the separatory funnel, the process was repeated until the bottom DCM layer is completely clear, and the DCM solution was dried using rotary evaporator to obtain the DCM extract. Using the same steps, EtAc extract was obtained.

Percentage yield was calculated by:

$$\%Y = \frac{W1}{W2} \times 100\% \quad \text{(Eq. 1)}$$

Where W1 is plant extract obtained and W2 is weight of plant sample before extraction

B. T. serrulatus

Essential oil (EO) from *T. serrulatus* was extracted using hydrodistillation in a Clevenger apparatus where the total of 300g of fresh leaves was immersed fully in distilled water for 3 h. The water layer at the bottom was first drained to separate from the oil. The oily sample was treated with anhydrous sodium sulphate (Na_2SO_4) to remove the remaining trace water. The obtained EO was stored at 4 °C for further analysis (Goudjil *et al*., 2020).

3.3. Phytochemical analysis

3.3.1. Qualitative Phytochemical screening of methanol crude extract

Phytochemical screening was according to the following protocols (Agbor *et al*., 2020; Singh *et al*., 2021; UC & Nair, 2013).

A. Terpenoids (Salkowski Test): Three drops of chloroform were added to the plant extract, followed by the careful addition of concentrated sulfuric acid. The formation of a yellow or reddish-brown color at the interface of the two layers indicates the presence of terpenoids.
B. Flavonoids (Shinoda Test): The plant extract was mixed with magnesium ribbon and a few drops of concentrated hydrochloric acid. The formation of a pink, red, or violet color indicates the presence of flavonoids.
C. Tannins (Ferric Chloride Test): Three drops of 5% ferric chloride solution were added to the plant extract. The formation of a bluish-black or greenish-black color indicates the presence of tannins.
D. Saponins (Froth Test): The plant extract was vigorously shaken with water in a test tube. The formation of a persistent froth that lasted for at least 10 min indicates the presence of saponins.
E. Alkaloids (Mayer's Test): Three drops of Mayer's reagent (containing potassium mercuric iodide) were added to the plant extract. The formation of a cream-colored or yellow precipitate indicates the presence of alkaloids.
F. Steroids (Salkowski Test): Three drops of concentrated sulfuric acid were added to the plant extract. The formation of a reddish-brown color indicates the presence of steroids.
G. Phenols (Ferric Chloride Test): A few drops of 5% ferric chloride solution were added to the plant extract. The formation of a bluish or greenish color indicates the presence of phenols.

3.3.2. Phytochemical screening of *T. serrulatus* EO

The phytochemical screening of *T. serrulatus* EO was done by UV spectrophotometry. A 100µl of oil was diluted with 3 ml chloroform and UV range 200 nm – 400 nm was used for determining natural compounds (Ammar *et al*., 2017; Urbano *et al*., 2006).

Terpenes have a UV absorption maxima in the 210-220 nm range. Phenols have a UV absorption maxima in the 270-280 nm range. Flavonoids have a UV absorption maxima in the 290-300 nm range. Coumarins have a UV absorption maxima in the 310-320 nm range. Alkaloids have a UV absorption maxima in the 330-340 nm range (Gierschner *et al*., 2012).

3.4. GC-MS analysis

GC-MS analysis of plant extracts was performed by a GC (7890B, Agilent Technologies, USA) coupled with an MS (5977A Network, Agilent Technologies). The GC had an HP-5MS column (30mm × 250 µm internal diameter (i.d.) and 0.25 µm). Helium was used as a carrier gas with 4 min solvent delay and splitless injection/purge time of 1.0 min with different flow rate and runtime (Table 2).

Table 2. GC-MS specifications for identification of compounds present

Extract	**Temperature raise**	**Flow rate**	**Run time**
***T. serrulatus* EO**	100 ºC to 280 ºC	1 ml/ min	45 min
***C. edulis* (DCM)**	110 ºC to 300 ºC	1.2 ml/min	33 min
***C. edulis* (EtAc)**	110 ºC to 300 ºC	1.2 ml/min	33 min

Mass spectra was recorded in an electron-impact mode, with ionization energy of mode at 70 eV, scanning the 33-550 m/z range (Gülçin, Küfrevioğlu, Oktay, & Büyükokuroğlu, 2004). The volatile compounds in the oil were identified by comparing the mass spectra of the compounds in the oils with those in the database of NIST11 GC-MS libraries (Gambaro *et al*., 2012; Hanuš *et al*., 2008).

3.5. Antibacterial activity test

The agar disk diffusion method was employed to assess the antibacterial properties of extracts of both plants against the four pathogenic bacterial strains, namely *P. aeruginosa* (ATTC27853), *E. coli* (ATTC25922), *S. aureus* (ATTC25923), and *S. pyogenes* (ATTC12204), respectively. The crude extracts were prepared in four sets of dilutions: 1000, 500, 250, and 125 µg/ml (for the crude extracts) by dissolving in 10% DMSO and the essential oil was prepared in three dilutions 40, 20, 10 µl/ml respectively by dissolving in 10% DMSO, respectively.

To quantify the bacterial population, a 0.5 McFarland standard was prepared by combining 0.05 ml of 1 percent $BaCl_2$ and 9.95 ml of 1 percent H_2SO_4 in distilled water. The bacterial

strains were adjusted to a concentration of 1.5×10^6 colony forming units (CFU/ml) and 100 µl of bacterial inoculum was spread on Mueller-Hinton agar.

Sterile filter paper disks with a diameter of 6 mm (Whatman No. 1) were prepared and soaked with the diluted extracts. Disks were then gently and evenly placed on the surface of the agar. After 15-30 min, the plates were incubated overnight at 37°C. Zone of inhibitions were measured in mm. Ciprofloxacin at 30µg/ml was used as a positive control and 10% DMSO was used as negative control, respectively. %inhibition was calculated relative to the control which was taken as 100%.

3.6. Antioxidant activities

Radical Scavenging Activity (%RSA) of both plant extracts were determined by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) method by measuring the decrease in absorbance of DPPH solution at 517 nm. The DPPH solution (0.1 mM) was prepared in methanol by dissolving 0.04 g DPPH in 100 ml methanol. The solution was kept in darkness for 30 min to complete the reaction. Four sets of concentrations were prepared for the extracts at 1000, 500, 250 and 125 µg/mL and same concentrations of ascorbic acid were used as a standard and sample free DPPH solution was used as a negative control, respectively. After mixing 1 ml of DPPH solution (0.04 mg/mL) with 3 ml of prepared samples, the mixture was incubated at room temperature in the dark for 30 min and the absorbance was measured at 517 nm. The %RSA was calculated according to the following formula.

$$\% \, RSA = \frac{Ao - A1}{Ao} \times 100\% \qquad \text{(Eq. 2)}$$

Where, %RSA is percent radical scavenging activity, A0 is the absorbance of the blank DPPH and A1 is the absorbance of sample (Bondet *et al.*,1997).

3.7. Minimum inhibitory concentration (MIC)

3.7.1. MIC determination for MeOH, EtAc, and DCM extracts of *C. edulis*

The bacterial growth was assessed by a broth dilution method. The extracts were diluted in DMSO to achieve the concentrations of 1 mg/ml, 500 µg/ml, 250 µg/ml l, and 125 µg/ml. Diluted extracts (100 µl) were introduced into 10 ml of LB broth and 100 µl of bacterial inoculum was added from McFarland standard calibrated broth (5×10^5 CFU (colony forming units) /mL) to each test tube and grown for 24 hr at 37 ºC. Absorbance of the culture was measured at 600 nm. The MIC was determined as the lowest concentration of extract that inhibited visible growth of the organism (Trentin *et al*., 2013).

3.7.2. MIC determination for EO of *T. serrulatus*

TMIC was done according to Broth microdilution method in a 96 well microtiter plate. A 20 µl of EO was added in 1 ml to achieve a stock solution of 20 µl/ml. Then stock was half diluted with LB broth in a microtiter plate with 100µl each of broth and sample. After dilution 100 µl of broth was added to achieve a total of 200µl in each well. 10 µl of McFarland calibrated (5×10^6 CFU/ml) bacterial inoculum was added to each cell and grown for 24h at 37 ºC. Absorbance was measured at 600 nm. The MIC was determined as the lowest dose at which the wells showed no turbidity due to bacterial growth.

3.8. Minimum bactericidal concentration (MBC)

After 24 h of inoculation, the MBC was determined based on the subculture from MIC on Mueller Hinton agar media. A 100µl of inoculum corresponding to the concentrations of extracts from the next three higher concentrations starting from the MIC were taken and transferred to Mueller Hinton agar and the MBC of extract was deduced from the lowest concentration on the total inhibition of bacteria on the agar (Agbor *et al*., 2020).

3.9. Antibiofilm assay

The antibiofilm assay was performed by mixing different concentrations starting from MIC (500-62.5 µg/ml for *C. edulis* and 0.02-1.25 µg/ml for *T. serrulatus*) in bacterial culture media culture at an initial turbidity of 0.05 (5 × 105 CFU (colony forming units) /mL) in a 96-well microtiter plate (for EO) and 10 ml test tubes (for MeOH, DCM, and EtAc extracts). The mixtures were incubated for 48 h at 37 ºC. The planktonic cells were removed by gentle washing with sterile Phosphate-buffered saline (PBS) and then the adherent cells were stained with 1% crystal violet (CV) for 10 min and washed with PBS to remove the excess stain. After air drying, the CV bound to the biofilm was solubilized with 33% glacial acetic acid (Calà *et al*., 2015; Magana *et al*., 2018).

Using similar procedures, a control without any treatment was set for comparison to quantify the adherent cells and the absorbance of the CV solution was measured by UV spectrophotometer at OD_{590}.

The percent biofilm formation was calculated by

$$\text{Biofilm formation (\%)} = \frac{OD590sample}{OD590Control} \times 100\% \qquad \text{(Eq. 3)}$$

3.10. Anti QS assay: Pyocyanin assay

The overnight grown planktonic cultures (6 ml) of *P. aeruginosa* with different dilutions (sub MIC) of plant extracts were added and centrifuged (5,000 g for 5 min) with 2 ml of chloroform. The organic layer was separated and solubilized in 2 ml of 0.2 M HCl. Then the absorbance of each supernatant was measured at 520 nm. Control without any treatment was set as 100% and distilled water with HCl was used as a blank (Kachhadia *et al*., 2022; Tapia-Rodriguez *et al*., 2017).

The inhibition percentage was calculated as:

$$\% \text{ Pyocyanin inhibition} = \frac{OD520\ of\ control - OD520\ of\ treated}{OD520\ of\ control} \times 100\% \quad \text{(Eq. 4)}$$

3.11. *In-silico* molecular docking study of identified compounds

The 3D crystal structures of selected QS proteins (LasI, LuxS, LsrR, AgrC) were chosen and retrieved from protein databank and saved as PDB format. The structures of the test compounds identified were drawn using ChemDraw Ultra 8, a chemical drawing software (Mendelsohn, 2004). The energy of the ligand/compounds was minimized using ChemDraw3D Ultra 8, a molecular modelling software (Mendelsohn, 2004). This step helps to ensure that the structures are stable and energetically favourable.

The PDB format of the compounds was then treated using MGL, a software package for processing PDB files. This step involved adding Gasteiger charges, merging out non-polar hydrogens, and automatically adjusting the torsion angle of the ligand. The prepared compound file was finally saved as PDBQT in the working directory. PDBQT is a file format that is commonly used for storing molecular structures. Then by using The CB-dock protein-ligand docking method was used to automatically identify the binding sites. Molecular docking was performed with AutoDock Vina (Liu *et al*., 2020). The binding affinity of a compound to the protein is measured in kilocalories per mole (kcal/mol). The root mean square deviation (RMSD) of the compound's position compared to the best docking mode was measured in angstroms (Å).

3.12. Statistical analysis

The mean ± standard deviation (SD) of three replicates represented the experimental results. Statistical significance was determined by P values less than 0.05. Microsoft Excel 2010 SPSS statistical software was utilized for all analyses.

CHAPTER FOUR

4. RESULTS

4.1. Plant extraction Yields

The extraction yield represents the amount of target extract obtained from a given amount of plant material. It is an important parameter that reflects the effectiveness of the extraction process and the potential bioactivity of the extracts.

In this study, various extraction methods were employed to obtain extracts from *C. edulis* and *T. serrulatus* plants. The extraction yields were determined by calculating the ratio of the weight of the obtained extract to the weight of the initial plant material, expressed as a percentage (Table 3).

Table 3. Percentage extract yield

Plant	Extract	Yield (%)
*C. **edulis***	MeOH	20.6%
	DCM	7%
	EtAc	5%
T. serrulatus	EO	0.43%

MeOH; Methanol, DCM; Dichloromethane, EtAc; Ethyl acetate.

4.2. Phytochemical screening

Phytochemical screening assays were conducted to identify the chemical classes present in the MeOH crude extract of *C. edulis*. The results revealed the presence of alkaloids, flavonoids, tannins, steroids, phenols, and Terpenoids, respectively (Table 4, Figure 6).

Figure 6. Phytochemical screening result qualitative observation.

Table 4. Qualitative phytochemical screening result for MeOH extract of *C. edulis*; (+) positive result, (-) negative result.

S.No	Phytochemical	Test result
1	terpenoids	+
2	Flavonoid	+
3	Tannins	+
4	Alkaloids	+
5	Phenols	+
6	Steroids	-

4.3. Phytochemical screening of *T. serrulatus* EO

The composition of plant extracts can be complex, containing a wide variety of compounds. The EO of *T. serrulatus* was monitored in the wavelength range of 200-400 nm. The different peaks in the range of wavelengths indicate the presence of different phytochemicals (Figure 7).

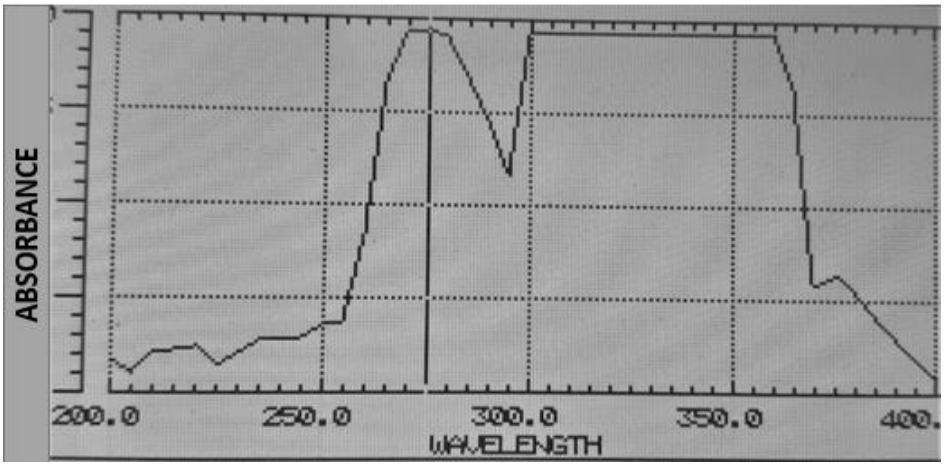

Figure 7. Phytochemical screening of the EO of *T. serrulatus*. Spectra was obtained in the range of 200-400 nm. The peaks were interpreted for the presence of different compounds.

Terpenes have a UV absorption maxima in the 210-220 nm range because they have conjugated pi systems while phenols have a UV absorption maxima in the 270-280 nm range due to the presence of aromatic rings. The flavonoids have a UV absorption maxima in the 290-300 nm range due to hydroxyl groups. The coumarins have a UV absorption maxima in the 310-320 nm range because of the presence of a coumarin ring. The alkaloids have a UV absorption maxima in the 330-340 nm range due to the presence of nitrogen atoms (Gierschner *et al.*, 2012).

4.4. GC-MS analysis

The GC-MS analysis was performed using standard protocols and appropriate analytical techniques. Due to the polarity of MeOH extract, the compatibility is poor with the columns used. Because GC coupled with an MS provides non-polar and volatile compounds. Therefore, it should not be used for MeOH extract quantitative and qualitative analysis as MeOH extracts mostly contain polar compounds. But unlike MeOH extract, DCM and EtAc extract are compatible with GC coupled with an MS (Bristow *et al*., 2023). DCM and EtAc extracts were subjected to GC-MS analysis. The chromatograms were generated for the different solvents used (DCM, EtAc and EO) (Figure 8). The major compounds were identified based on the intensity of the peaks and are summarized in Table 5.

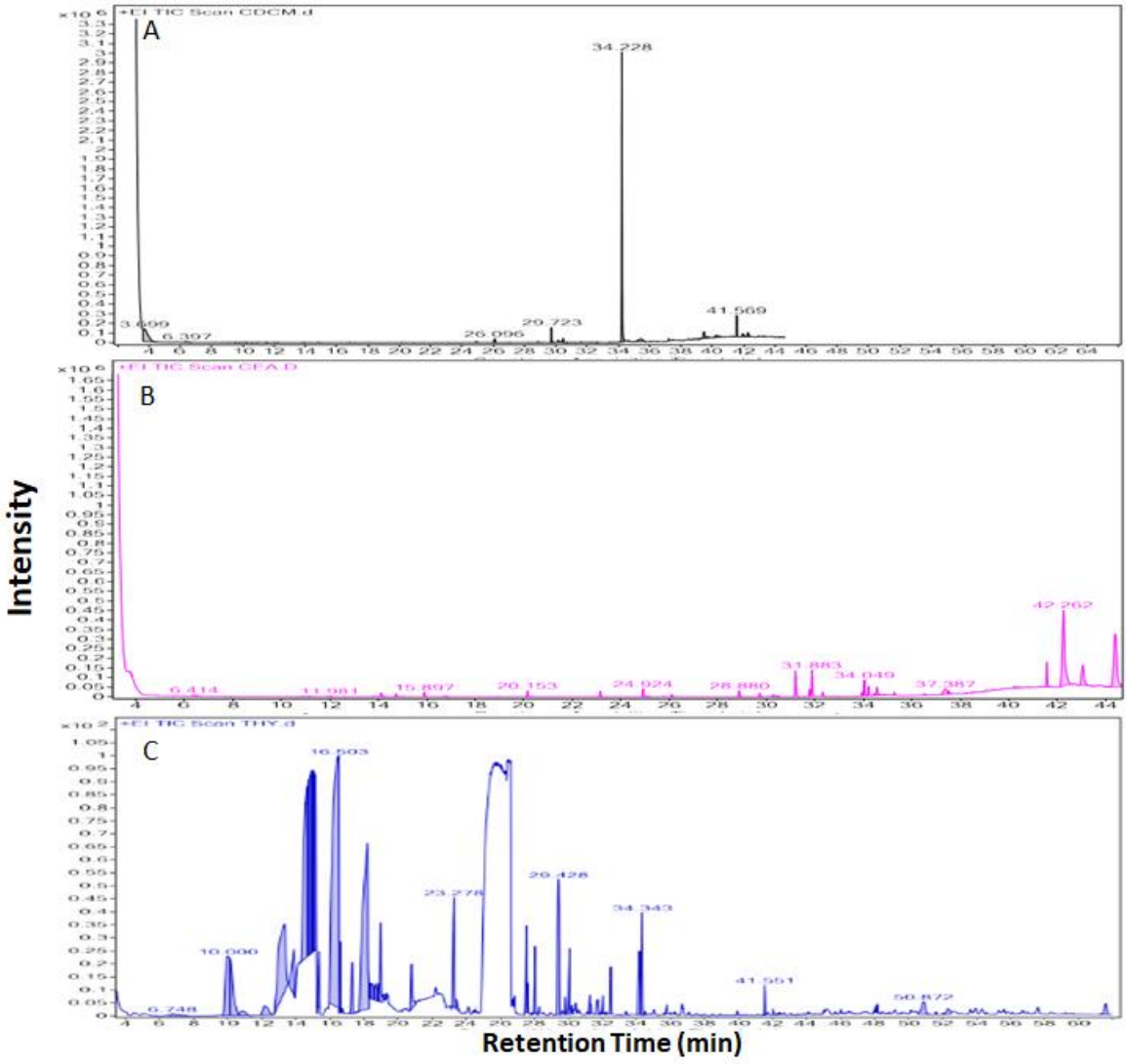

Figure 8. Chromatogram obtained by the GC-MS analysis **A**: DCM extract of *C. edulis*; **B**: EtAc extract of *C. edulis*; **C**: EO of *T. serrulatus*.

In the DCM chromatogram, phytol was identified as the major compound at 60.3% followed by Benzyl_carbazate at 20% and squalene at 4.4%, respectively. The EtAc chromatogram identified majorly (24R)-Stigmast-5-en-3.beta at 33%, β-Amyrin at 28%, squalene, α-Tocopheryl acetate at 4% each, respectively. In case of EO of *T. serrulatus*, 1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)- was identified as the major compound at 35% followed by p-Cymene at 2% and Carvacrol methyl ether at 1% respectively. Carvacryl acetate and 3-Octanol were also identified at 1% and 2%, respectively.

Table 5. Major compounds identified using GC-MS analysis which are >1%

Extract	**Compound**	**RT(min)**	**MF**	**Amount (%)**
DCM	phytol	34.228	C20H40O	60.3%
	squalene	41.569	C30H50	4.4%
	Neophytadiene	29.723	C20H38	2.8%
	5-Benzyl-2,3-dimethylpyrazine	26.096	C13H14N2	1%
	Hydrazinecarboxylic acid (Benzyl_carbazate)	3.699	C8H10N2O2	20%
EtAc	(24R)-Stigmast-5-en-3.beta.-ol	42.262	C29H50O	33%
	Squalene	41.569	C30H50	4%
	β-Amyrin	44.393	C30H50O	28%
	α-Tocopheryl acetate	37.387	C31H52O3	4%
	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	31.208	C17H24O3	4%
EO	1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	16.503	C10H16	35%
	p-Cymene	15.013	C10H14	2
	Carvacrol methyl ether	23.278	C11H16O	1
	3-Octanol	25.443	C10H14O	2
	Carvacryl acetate	28.037	C15H24	1%

RT: Retention time, MF: Molecular formula; %: amount in percentage.

4.5. Antibacterial activity test

4.5.1. Antibacterial activity of the *C. edulis* extracts

The antibacterial potential of the three *C. edulis* extracts (MeOH, DCM, and EtAc) were examined using the disk diffusion assay. Among the three extracts tested, EtAc and DCM exhibited the higher inhibitory activity against both the gram-positive and gram-negative bacterial strains tested at. The MeOH extract scored the lowest inhibition percentages (51% in *E.coli*) In general, the inhibitory activity of the extracts was found to be dose-dependent, where the zone of inhibition increased with the increasing concentrations. This trend was observed across different extracts and bacterial strains. The results were quantified by measurements of the zone of inhibitions in mm and presented in Table 6.

It was observed that the susceptibility of different bacterial strains varied. The gram-negative bacteria (*P. aeruginosa* and *E. coli*) showed similar levels of susceptibility to the extracts, while the gram-positive bacteria (*S. aureus* and *S. pyogenes* demonstrated higher susceptibility, in particular with EtAc and DCM extracts (Table 6). Due to the differential behavior of the positive control (Ciprofloxacin) against different bacteria, the zone of inhibition were not considered for comparative measurements. We chose to normalize the ZOIs taking control as the absolute value at 100%. These normalized values aided in a more meaningful comparison.

Table 6. Average inhibition zone and percentage inhibition of the *C. edulis* extracts

Extracts	**Mean diameter inhibition zone (in mm) ±SD**						**%inhibition**			
	Bacteria	**Cont**	**125**	**250**	**500**	**1000**	**125**	**250**	**500**	**1000**
MeOH	***P. aeruginosa***	27±0.6(%)	NA	NA	10±1.2	16±1.5	NA	NA	37%	59%
	E. coli	27±0.6	NA	NA	10±1.7	14±0.6	NA	NA	37%	51%
	S. aureus	30±1.7	NA	10±1	15.1±0.7	16±06	NA	37%	55%	59%
	S. pyogenes	26±0.6	8±1.53	9±1.2	15±1.2	16±2.1	29%	33%	55%	59%
DCM	***P. aeruginosa***	27±0.6	NA	NA	12±1.2	15±0.6	NA	NA	44%	55%
	E. coli	27±0.6	NA	NA	10±0.9	16±0.6	NA	NA	37%	59%
	S. aureus	30±1.7	11±0.6	15±1.7	16±1.7	16±1.2	36%	50%	53%	53%
	S. pyogenes	26±0.6	9±0.5	14±1.2	18±1.5	19±0.9	35%	53%	69%	73%
EtAc	***P. aeruginosa***	27±0.6	7±0.6	14±0.6	16±1.5	20±1.5	26%	52%	59%	74%
	E. coli	27±0.6	NA	9±1.5	13±0.6	15±1.2	NA	33%	48%	55%
	S. aureus	30±1.7	10±1	12±0.6	16±1.5	18±0.9	33%	40%	53%	60%
	S. pyogenes	26±0.6	7±0.6	15±1.2	17±1.2	20±1.2	27%	58%	65%	77%

Cont, Control; 125, 250, 500 and 1000 are concentrations in µg/ml; %inhibition is relative to the control which was taken as 100%; NA, No activity. Values are the round off to the nearest whole number of mean values of three independent experiments.

4.5.2. Antibacterial activity of *T. serrulatus* EO

The EO of *T. serrulatus* showed moderate to high inhibitory activity against the tested bacterial strains notably, it displayed stronger activity against the gram-positive bacteria , *S. aureus* (Sa) (57%) and *S. pyogenes* (Sp) (52%) compared to the gram-negative bacteria, *P. aeruginosa* (Pa) (47%) and *E. coli* (Ec) (41%), respectively (Figure 9, Table 7).

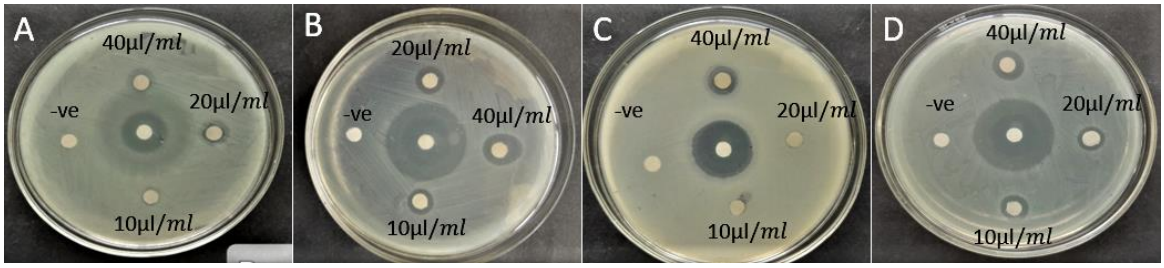

Figure 9. Antibacterial disk diffusion assay of EO extract against *P. aeruginosa* (A), *E. coli* (B), *S. aureus* (C), and *S. pyogenes* (D). Positive control (+) Ciprofloxacin 30 ug/ml was used and placed at the center, negative control (-ve) 10% DMSO.

Table 7. Average inhibition zone and percentage inhibition of the EO extract

		Mean diameter inhibition zone (in mm)				%inhibition		
Extract	**Bacteria**	**Control**	**10** µl/ml	**20** µl/ml	**40** µl/ml	**10** µl/ml	**20** µl/ml	**40** µl/ml
	P. aeruginosa	30	9	12	14	30%	40%	47%
	E. coli	29	11	12	17	38%	41%	59%
	S. aureus	23	7	8	13	30%	35%	57%
EO	***S. pyogenes***	29	13	12	15	45%	41%	52%

%inhibition is relative to the control which was taken as 100%. Values are the round off to the nearest whole number of mean values of three independent experiments.

4.6. Antioxidant activity tests

The antioxidation potential of the MeOH, DCM, and EtAc extracts of *C. edulis* and EO extract of *T. serrulatus* was assayed and evaluated using different concentrations. For this purpose, the DPPH assay was conducted for each extract sample solution. The results were expressed as %RSA compared to ascorbic acid (reference standard) (Table 8). The absorbance of the control DPPH solution, used as a baseline for all calculations, was measured to be 1.223. Additionally, Table 8 demonstrates the antioxidant activity of each sample, ranked by concentration. The antioxidant activity of the samples obtained through different extraction methods was found to be dependent on the dosage, increasing with higher concentrations.

Table 8. %RSA values of the extracts at different concentrations range.

Con (µg/ml)	MeOH	DCM	EtAc	EO	Standard
125	78.3	86.5	84.5	79.4	90.5
250	79.3	88.3	86.2	81.3	91
500	79.01	88.3	86.4	81.64	92.4
1000	80.2	89.18	87	82	94.7

Con, concentration of the extracts; Standard (Ascorbic Acid), respectively.

4.7. Minimum inhibitory concentrations (MIC)

The extracts of *C. edulis* were investigated by the broth dilution method to determine MIC. The MIC was defined as the lowest concentration of extract that inhibited visible growth of bacteria (Figure 10) and the absorbance was measured for the quantification of growth (Table 9).

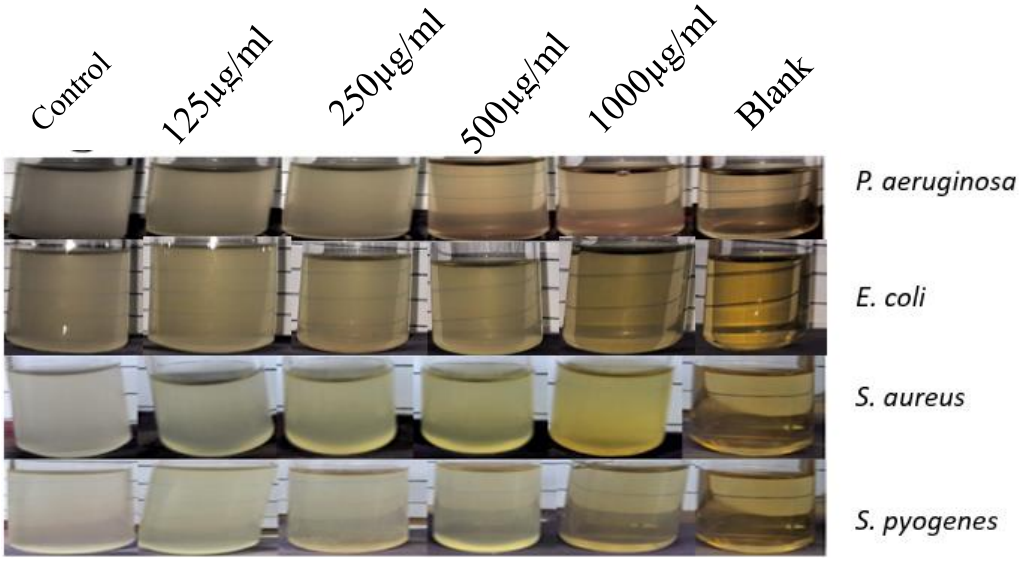

Figure 10. Visible growth inhibition of the MeOH extract on different bacteria.

Table 9. Average absorbance values of bacterial percentage growth

Extract	Average Absorbance values ± SD						%Growth			
	Bac	**Cont**	**125**µg/ml	**250** µg/ml	**500**µg/ml	**1000**µg/ml	**125** µg/ml	**250** µg/ml	**500** µg/ml	**1000** µg/ml
	P. aeruginosa	0.768±0.02	0.752±0.04	0.646±0.02	0.184±0.06	0.152±0.03	98%	84%	23%	19%
	E. coli	0.512±0.04	0.415±0.08	0.329±0.05	0.261±0.01	0.208±0.05	81%	64%	51%	40%
	S. aureus	0.951±0.02	0.77±0.07	0.581±0.05	0.507±0.07	0.501±0.09	81%	61%	53%	52%
MeOH	***S. pyogenes***	0.518±0.09	0.518±0.12	0.42±0.44	0.365±0.06	0.236±0.03	100%	81%	70%	46%
	P. aeruginosa	0.788±0.02	0.623±0.09	0.535±0.04	0.431±0.1	0.349±0.02	79%	68%	55%	44%
	E. coli	0.790±0.04	0.645±0.04	0.521±0.08	0.41±0.07	0.315±0.02	82%	66%	52%	40%
	S. aureus	0.910±0.02	0.632±0.05	0.563±0.1	0.482±0.03	0.461±0.04	69%	62%	53%	51%
DCM	***S. pyogenes***	0.593±0.09	0.483±0.01	0.378±0.06	0.302±0.08	0.229±0.01	82%	64%	51%	39%
	P. aeruginosa	0.768±0.02	0.709±0.02	0.677±0.04	0.307±0.07	0.253±0.05	92%	88%	40%	33%
	E. coli	0.512±0.04	0.496±0.04	0.425±0.07	0.3184±0.07	0.240±0.02	97%	83%	62%	47%
	S. aureus	0.951±0.02	0.826±0.05	0.598±0.0	0.397±0.09	0.380±0.02	87%	63%	42%	40%
EtAc	***S. pyogenes***	0.518±0.09	0.378±0.05	0.301±0.09	0.2014±0.07	0.204± 0.04	73%	58%	39%	39%

Concentrations corresponding to the highlighted absorbance values are taken as MIC

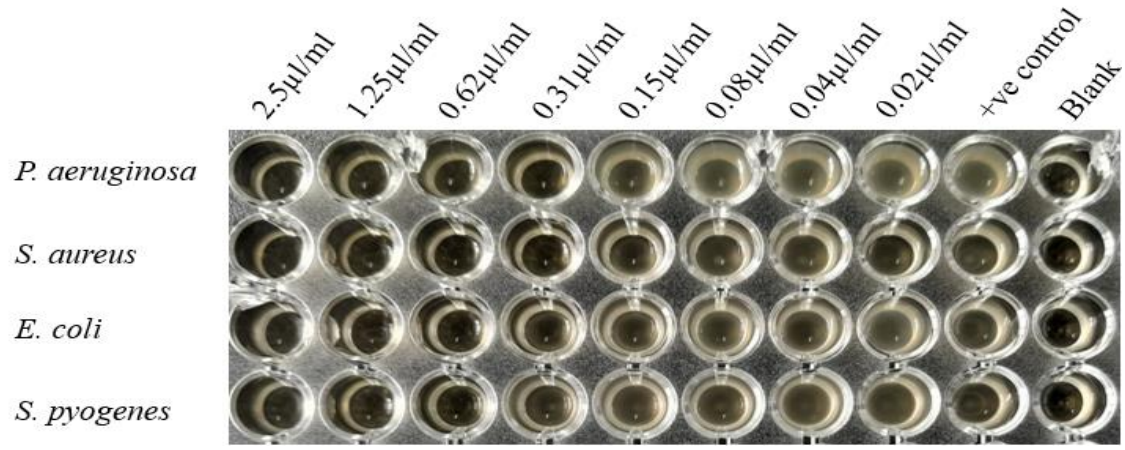

Figure 11. Determination of Minimum inhibitory concentration (MIC) of T. serrulatus EO against different bacterial strains. +ve control represent positive control (bacteria without any treatment) and blank contains only the broth without the bacteria.

As the concentration of the EO extract increased, the absorbance values decreased, indicating a decrease in bacterial growth (Figure 11). At a concentration of 0.02µl/ml, the percentage growth for *P. aeruginosa, E. coli, S. aureus,* and *S. pyogenes* were approximately 93.06%, 94.89%, 93.58%, and 90.84%, respectively (Table 10). At higher concentration (2.5 µl/ml), the percentage growth decreased to 23.43%, 40.51%, 29.05%, and, 27.20% for *P. aeruginosa, E. coli, S. aureus,* and *S. pyogenes* respectively.

Table 10. Average absorbance values and percentage growth (%G) of bacterial culture treated with different concentrations of EO extract.

	P. aeruginosa		***E.coli***		***S. aureus***		***S. pyogenes***	
	absorbance	**%Growth**	**absorbance**	**%Growth**	**absorbance**	**%Growth**	**absorbance**	**%Growth**
control	1.374 ± 0.101	100%	1.175 ± 0.209	100%	1.549 ± 0.185	100%	1.218 ± 0.229	100%
0.02µl/ml	1.28 ± 0.248	93.06%	1.117 ± 0.226	94.89%	1.448 ± 0.202	93.58%	1.108 ± 0.232	90.84%
0.04µl/ml	1.132 ± 0.273	82.33%	1.082 ± 0.271	92.00%	1.405 ± 0.214	90.64%	1.092 ± 0.203	89.67%
0.08µl/ml	0.834 ± 0.207	60.56%	1.067 ± 0.227	90.72%	1.379 ± 0.198	89.01%	0.992 ± 0.266	81.34%
0.15µl/ml	0.552 ± 0.158	40.18%	0.897 ± 0.216	76.38%	1.194 ± 0.22	77.16%	0.961 ± 0.212	78.98%
0.31µl/ml	0.453 ± 0.127	32.90%	0.777 ± 0.203	66.13%	0.96 ± 0.205	62.06%	0.887 ± 0.221	72.71%
0.62µl/ml	0.416 ± 0.123	30.21%	0.682 ± 0.186	58.09%	0.833 ± 0.24	53.78%	0.502 ± 0.143	41.17%
1.25µl/ml	0.364 ± 0.106	26.45%	0.681 ± 0.184	57.95%	0.743 ± 0.248	47.91%	0.481 ± 0.118	39.49%
2.5µl/ml	0.322 ± 0.109	23.43%	0.476 ± 0.165	40.51%	0.45 ± 0.139	29.05%	0.332 ± 0.195	27.20%

%G, percent growth relative to control which was taken as 100%. The highlighted numbers represent the visible growth inhibition and the corresponding concentration was taken as MIC. Concentrations corresponding to the highlighted absorbance values are taken as MIC.

4.8. Minimum bactericidal concentration (MBC)

The MBCs were determined by incubating the mixtures of MIC and higher concentrations on an agar media. The concentration at which no visible growth was observed visually was taken as MBC. The results are presented in Table 11. The MBC of EO and for *S. aureus* could not be determined as the MIC was more than the highest concentration tested *i.e.* 1mg/ml. Tolerance test values were also calculated which is defined as MBC divided by MIC. A tolerance test provides information about the potency of the test materials; higher values indicate bacteriostatic action, while lower values (<4) indicate bactericidal action (Govindasamy, Mydin, Sreekantan, & Harun, 2021).

Table 11. MIC and MBC values of extracts for bacteria *P. aeruginosa* (**Pa**), *E. coli* (**Ec**), *S. aureus* (**Sa**), and *S. pyogenes* (**Sp**).

	Bacteria	**MIC (mg/ml)**	**MBC (mg/ml)**	**MBC/MIC**
	P. aeruginosa	0.5	2	4
	E. coli	1	2	2
	S. aureus	>1	>2	-
MeOH	***S. pyogenes***	1	2	2
	P. aeruginosa	1	2	2
	E. coli	1	2	2
	S. aureus	>1	>2	-
DCM	***S. pyogenes***	1	2	2
	P. aeruginosa	0.5	1	4
	E. coli	1	2	2
	S. aureus	0.5	1	2
EtAc	***S. pyogenes***	0.5	2	2
	P. aeruginosa	0.31 µl	2.5 µl	8
	E. coli	0.62 µl	1.25 µl	2
	S. aureus	0.62 µl	>2.5 µl	-
EO	***S. pyogenes***	0.62 µl	1.25µl	2

4.9. Antibiofilm activity test

4.9.1. Antibiofilm activity of *C. edulis* Extracts

The formation of biofilm is an important adaptation by microbes against various stress factors such as antibiotics among many other stresses. The antibiofilm activity of the various extracts was performed by using CV staining method (Figure 12) as detailed in Materials and Methods.

In MeOH extract, as the concentration of the extract increased, the biofilm formation percentages of gram-positive bacteria decreased from 100% (no inhibition) at 62.5 µg/ml to 15% at 500 µg/ml (in *S. aureus*), indicating a dose-dependent increase in the inhibitory effect of the MeOH extract on biofilm formation. Similarly, in *S. pyogenes* the MeOH showed strong inhibition of biofilm formation at 28.6% (500 µg/ml). It appears that the MeOH extract might not have strong inhibitory properties against biofilm formation in gram-negative bacteria (*P. aeruginosa* and *E. coli*), as the biofilm formation percentages remained relatively constant even at higher concentrations (500 µg/ml) 43% in *P. aeruginosa* (Table 12).

In the case of DCM extract, the biofilm formation percentages decreased as the concentration increased, suggesting an inhibitory effect of the DCM extract on biofilm formation. At higher concentrations, the DCM extract displayed relatively higher inhibitory effects against biofilm formation in the tested bacterial strains. The DCM extract showed strong inhibitory properties against biofilm formation of gram-positive bacteria compared to gram-negative bacteria. Biofilm formation decreased from 98% at 62.5 µg/ml to 40.36% at 500 µg/ml in *S. aureus* and from 84.57% at 62.5 µg/ml to 38.14% at 500 µg/ml in *S. pyogenes*. In gram-negative bacteria the biofilm formation decreased from 82.21% at 62.5 µg/ml to 62.88% at 500 µg/ml in *P. aeruginosa* and from 90.55% at 62.5 µg/ml to 63.17% at 500 µg/ml in *E. coli.*

In EtAc extract, as the concentrations increased, the biofilm formation percentages generally decreased, indicating an inhibitory effect of the EtAc extract on biofilm formation. The EtAc extract demonstrated relatively higher inhibitory effects against biofilm formation at higher concentrations. Gram-positive bacteria (*S. aureus* and *S. pyogenes*) showed a decrease in biofilm formation from 84.60% at 62.5 µg/ml to 38.14% at 500 µg/ml in *S. aureus* and 63.3% at 62.5 µg/ml to 10.9% at 500 µg/ml in and *S. pyogenes* as the concentration of the extract increased. Gram-negative bacteria (*P. aeruginosa* and *E. coli*)

also showed a decrease in biofilm formation with increasing extract concentration. Biofilm formation decreased from 94.77% at 62.5 µg/ml to 38.95% at 500 µg/ml in *P. aeruginosa* and from at 100.20% to 61.47% at 500 µg/ml in *E. coli*.

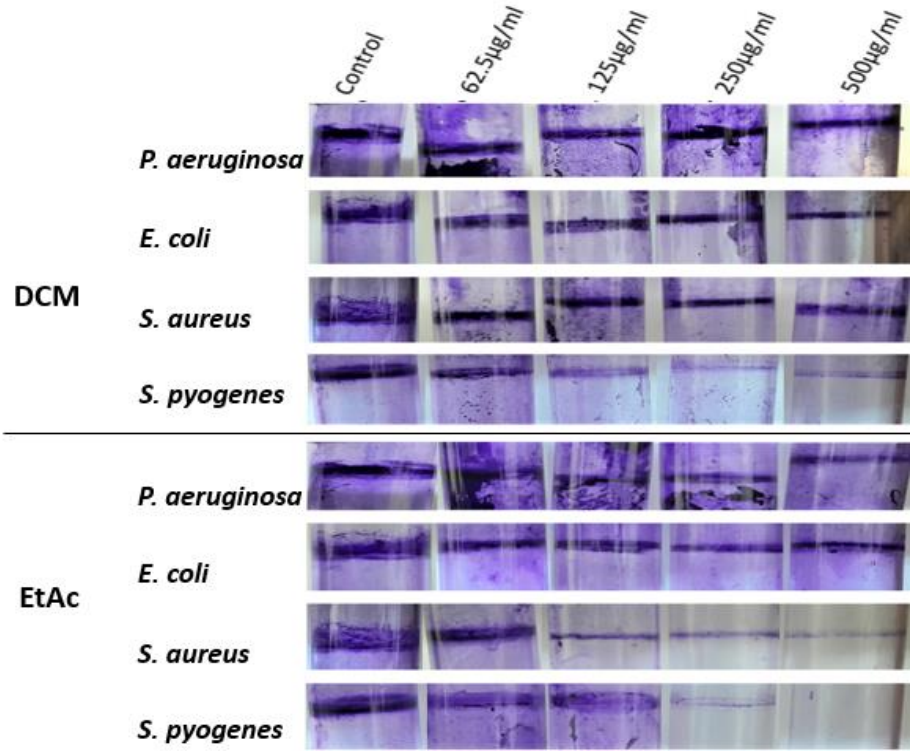

Figure 12. Qualitative observation of biofilm formation treated with different concentrations of DCM and EtAc extracts of *C. edulis* using CV staining method.

Table 12. Percentage biofilm formation of bacteria grown with different concentration of *C. edulis* extracts

Extracts	Bacteria	Concentration (µg/ml)				
		Control	62.5	125	250	500
MeOH	*P. aeruginosa*	100%	43.40%	43.40%	43.30%	43.40%
	E. coli	100%	87.50%	68.80%	64.60%	58.30%
	S. aureus	100%	100%	58.70%	36.50%	15.00%
	S. pyogenes	100%	90.10%	57.10%	33.00%	28.60%
DCM	*P. aeruginosa*	100%	82.21%	67.08%	62.88%	62.88%
	E. coli	100%	90.55%	78.12%	74.26%	63.17%
	S. aureus	100%	98.08%	94.61%	55.18%	40.36%
	S. pyogenes	100%	84.57%	80.08%	42.60%	38.14%
EtAc	*P. aeruginosa*	100%	94.77%	69.41%	68.09%	38.95%
	E. coli	100%	100.20%	86.87%	70.51%	61.47%
	S. aureus	100%	84.60%	80.08%	42.52%	38.14%
	S. pyogenes	100%	63.34%	35.29%	12.45%	10.91%

The values represent the percentage inhibition of bacterial growth relative to the control.

4.9.2. Antibiofilm activity of *T. serrulatus* EO extract

The percentage biofilm formation (%BF) also decreases with increasing EO concentration, indicating that EO has a dose-dependent impact on biofilm formation (Figure 13). The highest EO concentration (1.25 µl/ml) resulted in reduced biofilm formation, ranging from 6.06% to 30.14% across the different bacteria.

The EO demonstrates a noticeable effect on inhibiting biofilm formation in *P. aeruginosa* .At lower concentrations, such as 0.02µl/ml, the percentage biofilm formation (%BF) remains high at 99.15%, indicating significant biofilm formation. However, as the EO concentration increases, the %BF decreases, suggesting inhibitory effect of EO on Pa biofilm formation. At the highest concentration tested (1.25µl/ml), the %BF is 14.68%, indicating a substantial decrease in biofilm formation compared to the control (Figure 14).

In *E. coli*, the EO also exhibits an inhibitory effect on the biofilm formation in *E. coli*, although the reduction in %BF is not as pronounced as in *P. aeruginosa*. Even at the highest concentration (1.25µl/ml), the %BF is 28.14%, indicating a moderate yet significant antibiofilm activity against *E. coli*. The results suggest that *E. coli* might be relatively less susceptible to the EO's antibiofilm effects compared to *P. aeruginosa* (Figure 14).

In *S.aureus*, The EO was observed to be highly effective in inhibiting biofilm formation in *S. aureus*. As the EO concentration increases, the %BF decreases, indicating a reduction in *S.aureus* biofilm formation. At the highest concentration tested (1.25µl/ml), the %BF is significantly low at 6.06%, demonstrating a strong antibiofilm activity of EO against S. aureus (Figure 14).

The EO demonstrates a dose-dependent effect on inhibiting biofilm formation in *S. pyogenes*. The %BF decreases as the EO concentration increases, indicating a reduction in *S. pyogenes* biofilm formation. At the highest concentration (1.25µl/ml), the %BF is 30.14%, suggesting a moderate antibiofilm activity against *S. pyogenes* (Figure 14).

Figure 13. Visual observation of biofilm ring formation of bacteria grown in 96-well microtiter plate wells treated with different concentrations of EO extract. Arrows indicate biofilm ring formation intensity difference in control and the highest concentration.

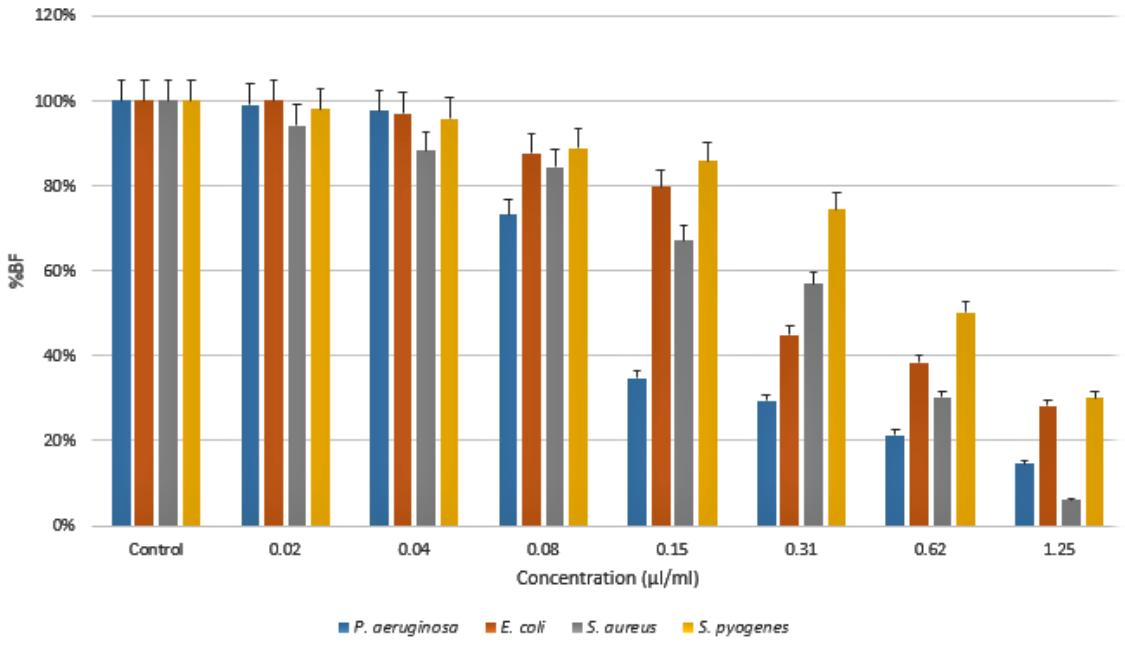

Figure 14. Graph representation of biofilm formation. Biofilm formation percentages (%BF) of EO extract at various concentrations.

4.10. Antiquorum sensing (QS) activity

Pyocyanin inhibition assay is a commonly used method to evaluate the inhibition of quorum sensing (QS) in the bacterium *P. aeruginosa*. QS is a cell-to-cell communication system that regulates the expression of various virulence factors in *P. aeruginosa*, including Pyocyanin production (J. W. Zhou *et al*., 2019).

Due to low yield of EO the Pyocyanin inhibition assay was done for the *C. edulis* MeOH, DCM and EtAc extracts. The results of Pyocyanin inhibition assay show that MeOH extract: At a concentration of 62.5 µg/ml, it showed 11.91% inhibition of Pyocyanin production. As the concentration increased, the inhibition also increased, reaching 44.34% at 500 µg/ml (Table 13). DCM extract exhibited a higher level of inhibition compared to the MeOH extract. At the lowest concentration (62.5 µg/ml), it showed 22% inhibition, which increased to 55.8% at 500 µg/ml. EtAc extract also exhibited inhibition of Pyocyanin production. At a concentration of 62.5 µg/ml, it showed 19.8% inhibition, which was further increased to 51.8% at 500 µg/ml.

Table 13. Pyocyanin inhibition of *C. edulis* extracts in percentage

Extract	**Concentration (µg/ml)**			
	62.5	**125**	**250**	**500**
MeOH	11.91%	29.15%	35.6%	44.34%
DCM	22%	35.3%	48.2%%	55.8%
EtAc	19.8%	31.2%	49.4%	51.8%

4.11. Molecular docking

3D crystal structures of representative QS proteins, LasI, LuxS, LsrR, AgrC, were chosen and retrieved from the Protein Data Bank (rcsb). These proteins play crucial roles in quorum sensing (QS), a cell-to-cell communication mechanism. To further investigate the interactions between these QS proteins and potential ligands, molecular docking was done. The binding affinity of a compound to the protein is measured in kilocalories per mole (kcal/mol) and has an inverse relationship with the binding. A lower affinity value indicates stronger binding. The root mean square deviation (RMSD) of the compound's position compared to the best docking mode is measured in angstroms (Å). It represents the structural deviation between different docking poses. Distance from Best Mode (RMSD U.B): The upper bound of the RMSD value, indicate the maximum structural deviation of the compound's position from the best mode. The RMSD values (root mean square deviation) indicate the degree of fit between the compounds and the proteins. The lower the RMSD value, the better the fit.

4.11.1. Molecular docking of compounds identified from DCM extract

Five major compounds were identified from the GC-MS analysis namely Phytol, Squalene, Neophytadiene, and Hydrazinecarboxylic acid, phenylmethyl ester (Benzyl_carbazate) (Figure 15, Table 15). The best interacting compound among the five compounds can be determined by considering the affinity values of molecular docking.

LasI is an enzyme found in *P. aeruginosa*, a gram-negative bacterium. It synthesizes and controls the production of the signaling molecule called N-(3-oxododecanoyl)-L-homoserine lactone (3OC12-HSL). LasI produces 3OC12-HSL by catalyzing the synthesis of the acyl-homoserine lactone (AHL) molecule from S-adenosylmethionine (SAM) and the corresponding fatty acid and LasR functions as a receptor for the signaling molecule N-(3-oxododecanoyl)-L-homoserine lactone (3OC12-HSL). When the concentration of 3OC12-HSL reaches a certain threshold, it binds to LasR, resulting in a conformational change in the protein (Schaefer *et al*., 2013). The molecular docking results revealed potential interactions between the LasI protein and the five major compounds: Phytol, Squalene, Neophytadiene, 5-Benzyl-2,3-dimethylpyrazine, and Benzyl_carbazate. Phytol and 5-Benzyl-2,3-dimethylpyrazine demonstrated the strongest binding affinity (-6.3 kcal/mol) in their respective best modes. Squalene and Neophytadiene also displayed favorable binding

affinities with values of -6.0 kcal/mol and -6.2 kcal/mol, respectively. Benzyl_carbazate exhibited moderate binding affinity with an affinity value of -5.8 kcal/mol.

LuxS and LsrR are two proteins involved in bacterial QS, which is a cell-to-cell communication mechanism commonly used by *E. coli*. LuxS is an enzyme that catalyzes the production of autoinducer-2 (AI-2) signaling molecule, which is a component of the methyl cycle and methionine metabolism. LsrR is a transcriptional repressor that binds to the promoter regions of genes involved in AI-2 uptake and metabolism which triggers an expression (Xue *et al*., 2009).

LuxS interacts with different ligands, including Phytol, Squalene, Neophytadiene, 5-Benzyl-2,3-dimethylpyrazine, and Benzyl_carbazate. Among these ligands, 5-Benzyl-2,3-dimethylpyrazine showed the highest affinity (-5.9 kcal/mol) for LuxS. This suggests that may have a strong binding interaction with LuxS, potentially affecting its enzymatic activity and interfering with the AI-2 signaling pathway.

AgrC is a receptor protein that plays a key role in the quorum sensing system of the gram-positive bacterium *S. aureus*. It is part of the Agr (accessory gene regulator) system, which controls the expression of various virulence factors and regulates the transition between colonization and infection (Novick, 2003).

Among the ligands, Squalene displayed the highest affinity (6.6 kcal/mol) for AgrC (Table 14). This suggests that Squalene may have a strong binding interaction with AgrC, potentially affecting its ability to recognize and respond to the signaling molecule, there by modulating the downstream gene expression.

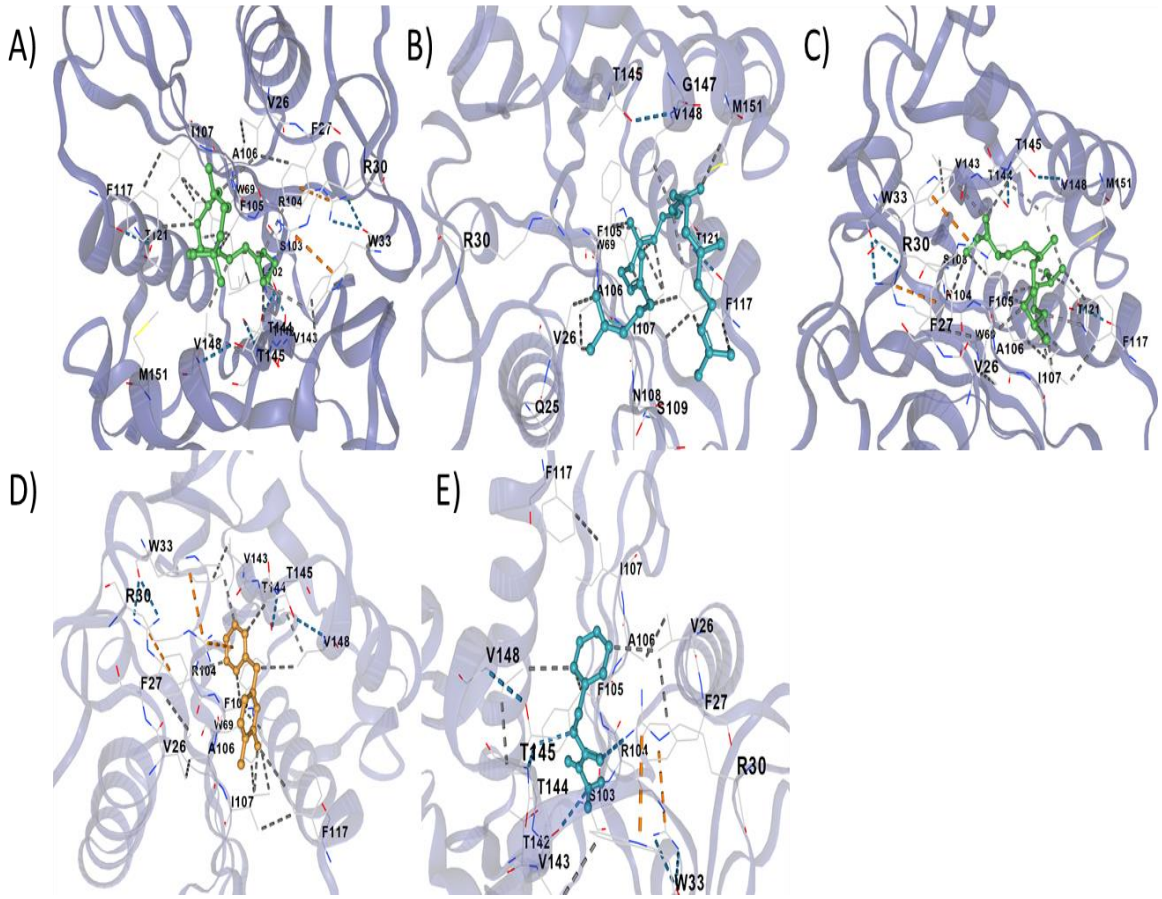

Figure 15. Representative image of 3D conformation interaction points of compounds identified (A; phytol, B; Squalene, C; Neophytadiene, D;5-Benzyl-2,3-dimethylpyrazine, E; Benzyl_carbazate) with LasI protein respectively.

Table 14. Summary of molecular docking result of major compounds of DCM extract

Protein	Ligand	Best Mode	Affinity (kcal/mol)	RMSD LB (Best Mode)	RMSD UB (Best Mode)	RMSD LB(Farthest Mode)	RMSD UB (Farthest Mode)
LasI	Phytol	1	-6.3	0.000	0.000	4.542	7.076
	Squalene	1	-6.0	0.000	0.000	2.493	6.003
	Neophytadiene	1	-6.2	0.000	0.000	2.517	5.964
	5-Benzyl-2,3-dimethylpyrazine	1	-6.3	0.000	0.000	3.617	4.666
	Benzyl_carbazate	1	-5.8	0.000	0.000	4.545	5.374
LuxS	Phytol	1	-5.2	0.000	0.000	1.932	3.598
	Squalene	1	-5.6	0.000	0.000	1.775	5.639
	Neophytadiene	1	-5.0	0.000	0.000	4.522	9.349
	5-Benzyl-2,3-dimethylpyrazine	1	-5.9	0.000	0.000	1.652	5.757
	Benzyl_carbazate	1	-5.4	0.000	0.000	5.214	6.031
LsrR	Phytol	1	-5.2	0.000	0.000	2.882	5.966
	Squalene	1	-6.9	0.000	0.000	1.946	5.771
	Neophytadiene	1	-5.2	0.000	0.000	2.122	4.772
	5-Benzyl-2,3-dimethylpyrazine	1	-6.0	0.000	0.000	1.642	1.777
	Benzyl_carbazate	1	-6.0	0.000	0.000	9.118	11.110
AgrC	Phytol	1	5.7	0.000	0.000	1.554	2.101
	Squalene	1	-6.6	0.000	0.000	2.691	6.016
	Neophytadiene	1	-5.6	0.000	0.000	1.304	2.226
	5-Benzyl-2,3-dimethylpyrazine	1	-6.1	0.000	0.000	2.543	6.099
	Benzyl_carbazate	1	-5.2	0.000	0.000	7.172	7.474

4.11.2. Molecular docking of compounds identified from EtAc extract

Five major compounds were identified from the GC-MS analysis of EtAc, namely (24R)-Stigmast-5-en-3.beta.- ol, Squalene, β-Amyrin, α-Tocopheryl acetate, 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione (Figure 8, Table 5). The best interacting compound among the five compounds can be determined by considering the affinity values of molecular docking.

Ligand (24R)-Stigmast-5-en-3.beta.-ol showed consistent docking across all four proteins. It exhibited relatively high affinity scores, ranging from -7.2 kcal/mol to -7.4 kcal/mol (Table 15). However, the RMSD values for the farthest docking mode ranged from 2.016 Å to 13.571 Å, suggesting potential conformational variability.

Squalene, Similar to (24R)-Stigmast-5-en-3.beta.-ol, this ligand demonstrated consistent docking across all proteins. The affinity scores ranged from -6.0 kcal/mol to -6.9 kcal/mol. The farthest docking mode had RMSD values ranging from 1.775 Å to 6.003 Å.

β-Amyrin displayed relatively high affinity scores, ranging from -7.3 kcal/mol to -8.4 kcal/mol. The farthest docking mode showed RMSD values ranging from 1.636 Å to 4.134 Å. Overall, the results suggest that all the ligands have the potential to bind to the QS proteins.

Table 15. Summary of molecular docking result of major compounds of EtAc extract

Protein	Ligand	Best Mode	Affinity (kcal/mol)	RMSD LB (Best Mode)	RMSD UB (Best Mode)	RMSD LB(Farthest Mode)	RMSD UB (Farthest Mode)
LasI	(24R)-Stigmast-5-en-3.beta.- ol	1	-7.2	0.000	0.000	13.571	17.371
	Squalene	1	-6.0	0.000	0.000	2.493	6.003
	β-Amyrin	1	-7.3	0.000	0.000	2.314	2.773
	α-Tocopheryl acetate	1	-6.7	0.000	0.000	2.064	3.803
	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	1	-6.7	0.000	0.000	2.685	5.772
LuxS	(24R)-Stigmast-5-en-3.beta.- ol	1	-7.4	0.000	0.000	4.497	7.144
	Squalene	1	-5.6	0.000	0.000	1.775	5.639
	β-Amyrin	1	-7.5	0.000	0.000	1.751	4.134
	α-Tocopheryl acetate	1	-5.5	0.000	0.000	3.640	8.805
	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	1	-5.7	0.000	0.000	2.288	3.686
LsrR	(24R)-Stigmast-5-en-3.beta.- ol	1	-7.3	0.000	0.000	5.122	7.666
	Squalene	1	-6.9	0.000	0.000	1.946	5.771
	β-Amyrin	1	-8.4	0.000	0.000	1.636	3.549
	α-Tocopheryl acetate	1	-6.5	0.000	0.000	10.565	16.214
	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	1	-6.4	0.000	0.000	2.584	4.989
AgrC	(24R)-Stigmast-5-en-3.beta.- ol	1	-7.3	0.000	0.000	2.016	3.566
	Squalene	1	-6.6	0.000	0.000	2.691	6.016
	β-Amyrin	1	-8.0	0.000	0.000	3.702	3.275
	α-Tocopheryl acetate	1	-7.4	0.000	0.000	2.187	6.309
	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	1	-7.4	0.000	0.000	2.842	4.776

5. Discussion

The development of antibiotic resistance is becoming a major menace in the treatment of infectious diseases. Therefore, this has posed urgent need of new treatment strategies leading to search and discover alternative medicines than are commonly used which have become ineffective. In this vein, natural compounds from plants have proven to be effective sources of molecules to combat the antibiotic-resistant microbes due to the presence of active compounds that have shown promising therapeutic potential. In this study, we have chosen two plants, *C. edulis* and *T. serrulatus*, to investigate their potential as antibacterial and antioxidant agents.

The leaves of *T. serrulatus* were subjected to hydrodistillation to obtain essential oil with a yield of 0.43%. These results were found to be in line with the previous reports (Olawuwo *et al*., 2022). The qualitative phytochemical screening of the MeOH extracts showed the presence of phenols, alkaloids, flavonoids, terpenoids, tannins and saponins, however, steroids were not detected, respectively (Figure 6, Table 4). The phytochemical screening of the EO of *T. serrulatus* was found to contain terpenes, phenols, flavonoids, coumarin and alkaloids, respectively. In general, these classes of compounds are known to possess antibacterial as well as antioxidant properties and have been found to be effective against microbes such as pathogens causing diseases, food spoilage, poultry and so on (Damtie & Mekonnen, 2020; Galovičová *et al*., 2021; Umaru *et al*., 2019).

The GC-MS analysis was effective to identify and quantify the compounds present in the three extracts analysed DCM and EtAc (*C. edulis*) and EO (*T. serrulatus*). The major compounds found in the DCM extracts were phytol at 60.3%, Benzyl_carbazate at 20% and squalene at 4.4%, respectively (Table 5). Phytol has been reported to be present in various species like *Glinus oppositifolius, Abutilon indicum* and *Borassus flabellifer* (Ragasa *et al.*, 2015; Sibuyo *et al*., 2017; Thakor *et al*., 2016). Phytol is known to have antibacterial, antibiofilm, and antioxidant activities (Carocho & CFR Ferreira, 2013; Islam *et al*., 2018). The major compounds found in the EtAc extract were (24R)-Stigmast-5-en-3.beta at 33%, β-Amyrin at 28%, squalene, α-Tocopheryl acetate at 4%. These compounds have been reported in other plant species (Dayal *et al*., 1984; Rogge *et al*., 2006). α-Tocophery acetate is a form of vitamin E and is found in many foods such as vegetable oils, nuts, and seeds (Duncan & Suzuki, 2017) and have been reported to have good antioxidant properties (Yamauchi, 1997).

The major compounds found in EO of *T. serrulatus* were 1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)- at 35% followed by p-Cymene at 2% and Carvacrol methyl ether at 1% respectively (Table 5). Carvacryl acetate and 3-Octanol were also identified at 1% and 2%, respectively this compounds have been reported in *Thymus* and other plant species (Galovičová *et al*., 2021; Merchán *et al*., 1999; Najeeb Ur Rehman *et al*., 2021; Tapia-Rodriguez *et al*., 2017). One of the abundant compound in EO, 1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl) is found in many essential oils and have been shown to have antibacterial, antibiofilm and antioxidant activity (Damtie & Mekonnen, 2020; Mollica *et al*., 2022)

The antibacterial activity of the extracts were analyzed by the disk diffusion assay that allows measurements of the inhibition of bacteria to form a clear zone around the disk impregnated with the compounds being tested. The measured area of the zone of inhibition indicates the strength of inhibitory potential of the samples. Due to the differential behavior of the positive control (Ciprofloxacin) against different bacteria that may be due to the resistance of the bacteria towards the antibiotic, the zone of inhibition were not considered for comparative measurements. We chose to normalize the ZOIs taking control as the absolute value at 100%. These normalized values aided in a more meaningful quantification and comparison. The quantification was done in terms of relative inhibition with respect to the zone of inhibition of the control.

In general, all the extracts tested were able to inhibit the bacteria (both gram-positive and gram-negative) albeit with varying degrees of inhibition (Table 6). The EtAc extract showed inhibition in the range of 55-77% but did not show clear inhibition between gram-positive and gram-negative. The gram-positive bacteria showed inhibition of 60-77% while the gram-negative bacteria showed inhibition of 55-59%, respectively. The DCM extract showed inhibition in the range of 53-73% and also indicated difference in the susceptibility of gram-positive and gram-negative. The gram-positive bacteria were more susceptible (53-73%) while the gram-negative bacteria were less susceptible (55-59%), respectively. The gram-positive bacterial cell wall is composed of teichoic acids that partially imparts a negative charge to the cell surface and facilitate passage of positively charged molecules, interact with charged protein in the cell wall and thereby plausibly rupture the cells resulting in higher antibacterial activity of such molecules. On the other hand, the cell wall composition of gram-negative bacteria is complex and consists of a second membrane that restricts the

penetration of molecules and thereby plausibly imparts resistance to antibacterial agents. The MeOH extract showed the least inhibition (51-59%) compared to DCM and EtAc and did not indicate difference in the susceptibity behavior of the gram-positive and gram-negative. The lower inhibition of MeOH extracts may be due to the lower concentration of the molecules in the extracts. The DCM and EtAc extracts underwent a second extraction step which may have concentrated the molecules in the extraction environment of the solvent. Similarly, the EO also showed a general susceptibility of bacteria with no discrimination as both the gram-positive and gram-negative bacteria showed similar susceptibility (52-57% for gram-positive and 47-59% for gram-negative).

The antioxidant activity test assay of the sample extracts demonstrated the effective potential of each extract to scavenge free radicals. The DPPH assay method employed to test the activity of the samples recorded a range of activity between 78.3 to 89.18 % RSA across the different extracts. All the extracts tested have displayed an increasing radical scavenging activity with an increase in concentration. However, the lower range of the activity also require investigations for a safe conclusion. These values are comparable to the reported values (Carocho & CFR Ferreira, 2013). Compared to the activity of standard ascorbic acid used in the study which has shown 90.5 - 94.7 % RSA at different concentrations (ranging from 125µg/ml up to 1mg/ml), the extracts have displayed comparable activity. Several studies have reported the antioxidant property from extracts of different plants species and natural products of plants like *Basel, Glinus oppositifolius, Abutilon indicum* and *Borassus flabellifer* (Altemimi *et al*., 2017; Carocho & CFR Ferreira, 2013; Stagos, 2019).

The investigation and determination of MIC and MBC for antibacterial agents provides a holistic view about the potentiality and stoichiometric inhibitory actions. The MIC is the lowest concentration of the samples that inhibit visible growth of bacteria. In this study, the investigation of MIC was done by the broth assay by incubating different bacteria with different concentration of the various extracts. Post incubation, the samples were examined both visually and spectrophotometrically. The MIC values varied between 500 µg/ml -1 mg/ml for different bacterial strains without a clear indication of any difference between gram-positive and gram-negative bacteria. Since there was no visible inhibition of growth observed in *S. aureus* in the range of concentrations tested the MIC values were reported as >1 mg/ml. These values were found to be in agreement with the reported literature (Garcia *et al*., 2022). In the case of EO, the MIC values were found to be in the range of 0.31-0.62 µl/ml for different bacteria which is in agreement with the range reported earlier (Galovičová

et al., 2021). The MBC is defined as the concentration at which no visible growth of bacteria is observed. The MBC values for MeOH, DCM and EtAc extracts were found to be 200 mg/ml and 1.25-2.5 µl/ml foe EO, except *S. aureus*. A tolerance test provides information about the potency of the test samples; higher values indicate bacteriostatic action, while lower values (<4) indicate bactericidal action. In our study, the Tolerance values were found to be in the range of 2-4 that places all the extracts in the category of bactericidal agents. However, higher Tolerance value of 8 was recorded for *P. aeruginosa* against the EO that indicates towards bacteriostatic action (Govindasamy *et al*., 2021).

The formation of biofilm is an important adaptation by microbes against various stress factors such as antibiotics among many other stresses. The biofilms enable the bacteria to sustain antibiotics 10-1000 times more than the normal planktonic susceptible concentration. Therefore, it is important to investigate the potential of the isolated products/extracts to inhibit the formation of biofilms. In this study, it was found that the MeOH extract had a dose-dependent inhibitory effect on biofilm formation in gram-positive bacteria such as *S. aureus* and *S. pyogenes*. However, the MeOH extract did not exhibit strong inhibitory properties against biofilm formation in *P. aeruginosa*. The biofilm formation remained relatively constant at ~40% even at higher concentrations (500 µg/ml). These results are consistent with the results of other studies that have investigated the antibiofilm activity of methanolic extracts. In the report of (Biswas *et al.*, 2013), the MeOH extracts of guava leaves showed inhibitory activity against gram-positive bacteria, however, gram-negative bacteria were reported to be resistant to MeOH extract. It is known that the formation of biofilms is more pronounced in gram-negative bacteria than gram-positive bacteria that make them more resistant to antibiotics (Ruhal & Kataria, 2021). However, further detailed investigations may be required to elucidate the reasons of resistance. The DCM extract displayed stronger inhibitory effects against biofilm formation in gram-positive bacteria (38.14-40.36%) compared to gram-negative bacteria (62.88-63.17%), respectively. Similarly, the EtAc extract demonstrated effective inhibition of the biofilm formation in both gram-positive (10.91-38.14%) and gram-negative bacteria (38.95-61.47%). A lower inhibition (Biofilm formation 61.7%) was observed in *E. coli* which may be due to resistance by this specific gram-negative bacteria against the compounds in EtAc extract that require further investigation. The resistance demonstrated by gram-negative bacteria is in agreement with the general observation made for *Psidium guajava* and *Boletus edulis* extracts (Biswas *et al*., 2013; Garcia *et al*., 2022)

Overall, the results suggest that the three *C. edulis* extracts, particularly the DCM and EtAc extracts, have potential antibiofilm activity. The extracts demonstrated stronger inhibitory effects against biofilm formation in gram-positive bacteria compared with the gram-negative bacteria. These findings highlight the importance of further investigating the active compounds present in the *C. edulis* extracts and their mechanisms of action against biofilm formation. In general, the results of this experiment suggest that *C. edulis* extracts was a potential antibiofilm agent. Further studies are needed to determine the mechanism of action of these extracts and to optimize their use against biofilm-forming bacteria.

The results of the pyocyanin inhibition assay show that the MeOH, DCM, and EtAc extracts of *C. edulis* all have some inhibitory effect on pyocyanin production in *P. aeruginosa*. The MeOH extract had the lowest level of inhibition, ranging from 11.91% up to 44.34% at 500 μg/ml while the DCM extract had the highest level of inhibition ranging from 22% up to 55.8% at 500 μg/ml. The EtAc extract had an intermediate level of inhibition compared to the other extracts this results are consistent with other studies. In the study (Jain *et al*., 2017) MeOH extracts of different plants with >50% inhibition of the production of pyocyanin was observed. This shows that *C. edulis* extracts have the potential to inhibit the production of pyocyanin in *P. aeruginosa*.

Representative QS proteins, LasI, LuxS, LsrR, AgrC, were chosen and retrieved from the Protein Data Bank (rcsb). These proteins play crucial roles in quorum sensing (QS), a cell-to-cell communication mechanism. The DCM and EtAc extracts exhibited ligands with relatively high affinity scores for the target proteins involved in gram-negative and gram-postive bacterial QS (Schaefer *et al*., 2013; Xue *et al*., 2009). LasI, LuxS, and LsrR are representatives of gram-negative QS and AgrC is a representative of gram-positive bacterial QS (Novick, 2003). EtAc extract consistently showed higher affinity scores compared to the DCM extract for all three proteins of (LasI, LuxS, and LsrR).

The specific ligands with the highest affinity scores varied for each protein and extract. For the LasI protein, Phytol and 5-Benzyl-2,3-dimethylpyrazine exhibited the strongest binding affinity with values of -6.3 kcal/mol. Squalene and Neophytadiene also showed favorable binding affinities with values of -6.0 kcal/mol and -6.2 kcal/mol, respectively. Benzyl_carbazate displayed a moderate binding affinity with an affinity value of -5.8 kcal/mol. These results suggest that Phytol, Squalene, Neophytadiene, and 5-Benzyl-2,3-dimethylpyrazine have the potential to interact with LasI and may influence its activity in

the synthesis of the signaling molecule 3OC12-HSL. In the case of LuxS, 5-Benzyl-2,3-dimethylpyrazine exhibited the highest affinity (-5.9 kcal/mol), suggesting a strong binding interaction with LuxS. This interaction could potentially affect the enzymatic activity of LuxS and interfere with the AI-2 signaling pathway.

Regarding AgrC, Squalene displayed the highest affinity (6.6 kcal/mol). This suggests that Squalene may strongly bind to AgrC, potentially affecting its ability to recognize and respond to the signaling molecule and thereby modulating downstream gene expression. The EtAc extract, the major compounds identified were (24R)-Stigmast-5-en-3.beta.-ol, Squalene, β-Amyrin, α-Tocopheryl acetate, and 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione. The molecular docking results indicated potential interactions between these compounds and the same three proteins: LasI, LuxS, and AgrC.

(24R)-Stigmast-5-en-3.beta.-ol consistently showed relatively high affinity scores across all proteins, ranging from -7.2 kcal/mol to -7.4 kcal/mol. Squalene also demonstrated consistent docking across all proteins with affinity scores ranging from -6.0 kcal/mol to -6.9 kcal/mol. β-Amyrin displayed relatively high affinity scores ranging from -7.3 kcal/mol to -8.4 kcal/mol. These results indicate that all the identified compounds from the EtAc extract have the potential to bind to the QS proteins and may influence their activities.

In summary, the molecular docking analysis revealed potential binding interactions between the identified compounds from the DCM and EtAc extracts and key proteins involved in bacterial quorum sensing systems. The obtained affinity values shed light on the strength of these interactions and provide a basis for further experimental investigations into the effects of these compounds on bacterial signaling pathways and gene expression.

6. Conclusion

The development of antibiotic resistance has become a significant concern in the treatment of infectious diseases, necessitating the search for alternative medicines. Natural compounds from plants have shown promise in combating antibiotic-resistant microbes due to their active compounds with therapeutic potential. In this study, the potential antibacterial and antioxidant properties of two plants, *C. edulis* and *T. serrulatus*, were investigated. The extracts obtained from these plants exhibited a range of bioactive compounds. The extracts from *C. edulis*, obtained using different solvents, showed varying degrees of antibacterial activity against both gram-positive and gram-negative bacteria. The essential oil from *T. serrulatus* also exhibited antibacterial activity without discrimination between gram-positive and gram-negative bacteria. Furthermore, all the extracts demonstrated antioxidant activity, comparable to that of the standard ascorbic acid. The extracts from *C. edulis* showed potential as antibiofilm agents, particularly against gram-positive bacteria, while the *T. serrulatus* extracts displayed effective inhibition of biofilm formation in both gram-positive and gram-negative bacteria. The extracts of *C. edulis* also exhibited some inhibitory effect on pyocyanin production in *P. aeruginosa*, suggesting their potential as anti-quorum sensing agents. Molecular docking analysis further supported the binding affinity of the active compounds present in these extracts with QS proteins. These findings highlight the potential of *C. edulis* and *T. serrulatus* as sources of alternative medicines for combating antibiotic-resistant microbes and the need for further research to elucidate their mechanisms of action.

7. Recommendations

Based on the findings of this study, the following recommendations can be made:

- Further Investigation of Active Compounds: It is recommended to conduct further research to identify and isolate the active compounds present in the extracts of *C. edulis* and *T. serrulatus.* Understanding the specific compounds responsible for the observed antibacterial, antioxidant, antibiofilm, and quorum sensing inhibition activities can provide insights into their mechanisms of action and potential applications in combating antibiotic-resistant bacteria.
- Mechanistic Studies: In order to elucidate the mode of action of the extracts, additional mechanistic studies should be conducted. Investigating the impact of the extracts on microbial cell structures, biofilm formation, quorum sensing pathways, and gene expression can provide a deeper understanding of how these natural compounds interact with bacterial systems.
- Combination Studies: Considering the rise of antibiotic resistance, it would be valuable to explore the potential synergistic effects of the extracts with existing antibiotics. Combination studies can assess whether the extracts enhance the efficacy of antibiotics or help to overcome resistance mechanisms, potentially leading to more effective treatment options.
- In Vivo Studies: To validate the therapeutic potential of *C. edulis* and *T. serrulatus* extracts, further studies involving animal models should be conducted. In vivo studies can provide important information about the safety, pharmacokinetics, and efficacy of the extracts, laying the groundwork for their potential development as alternative therapies.

By following these recommendations, we can further explore the potential of *C. edulis* and *T. serrulatus* extracts as alternative therapeutic options, contribute to the fight against antibiotic resistance, and potentially improve patient outcomes in the treatment of bacterial infections.

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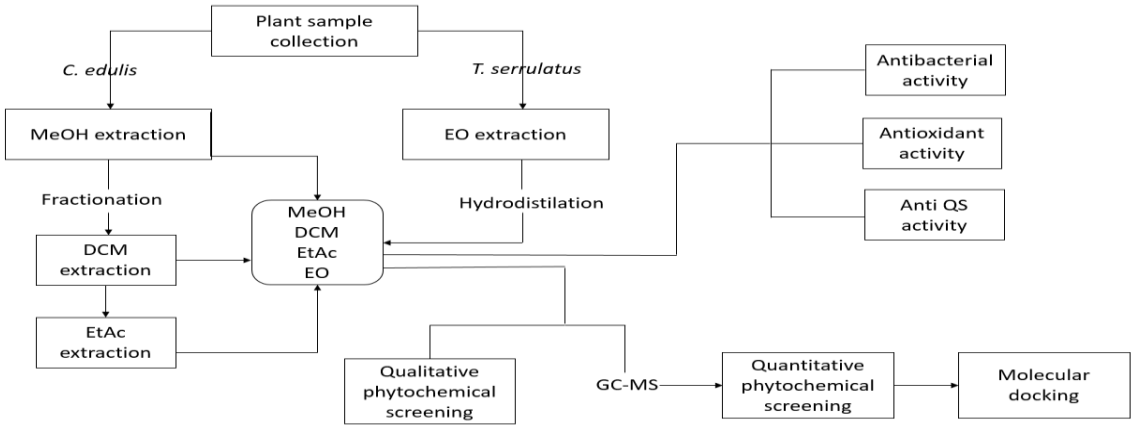
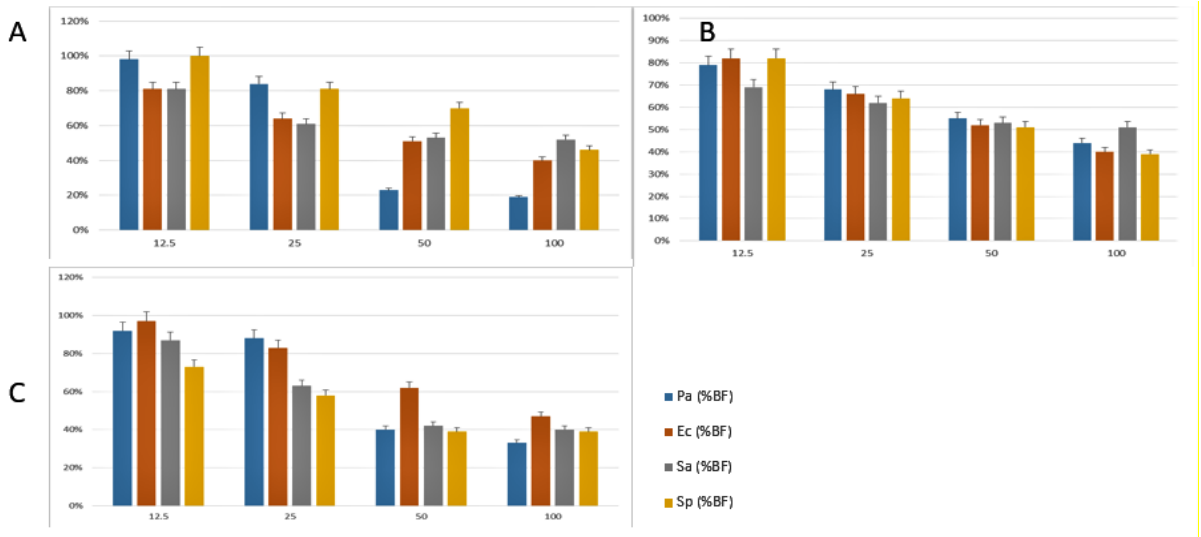
10. APPENDIX



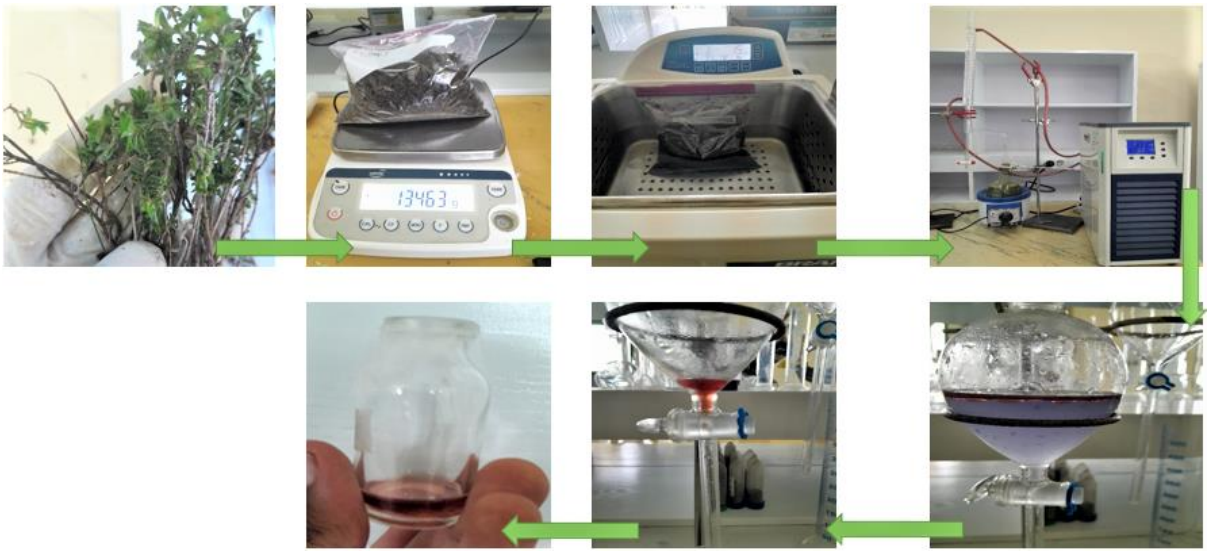
Appendix figure 1. Flowchart summary of the method used in this study



Appendix figure 2. Graphical representation for the percentage growth of *P. aeruginosa* (Pa), *E. coli* (Ec), *S. aureus* (Sa), and *S. pyogenes* (Sp) at various concentrations of *C. edulis* extracts. A; MeOH extract, B; DCM extract, and C; EtAc extract.

(APENDEX)

Appendix figure 3. Flowchart of methanolic extraction



Appendix figure 4. Flowchart of essential oil extraction