Phytochemical Composition and Evaluation of the Antimicrobial, Antioxidant, Antibiofilm, and Antiquorum Sensing Activities of *Acanthus polystachyus* Delile (Acanthaceae) Leaf Extracts.

By

Meron Getahun Teferi

A Thesis Submitted to the Department of Applied Biology School of Applied Natural Science

In partial fulfillment of the Requirements for the Degree of Master of Science in Applied Biology (Biotechnology)

Office of Graduate Studies

Adama Science and Technology University

June 2024

Adama, Ethiopia

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DECLARATION

I hereby declare that this Master's Thesis entitled **"Phytochemical Composition and Evaluation of the Antimicrobial, Antioxidant, Antibiofilm, and Antiquorum Sensing Activities of *A. polystachyus* Delile (Acanthaceae)Leaf Extracts."** is my original work. It has not been submitted for the award of any academic degree, diploma, or certificate in any other university. All sources of materials used in this thesis have been duly acknowledged through citation.

_____	_____	_____
Name of the student	Signature	Date

RECOMMENDATION OF ADVISORS

We, the advisors of this thesis, hereby certify that we have read the revised version of the thesis entitled **"Phytochemical Composition and Evaluation of the Antimicrobial, Antioxidant, Antibiofilm, and Antiquorum Sensing Activities of *A. polystachyus* Delile (Acanthaceae) Leaf Extracts."** prepared under our guidance by Meron Getahun Teferi submitted in partial fulfillment of the requirements for the degree of Mater's of Science in Applied Biology (Specialization in Biotechnology). Therefore, we recommend the submission of a revised version of the thesis proposal to the department following the applicable procedures.

_____	_____	_____
Major-Advisor	Signature	Date

APPROVAL OF ADVISORS

We, the advisors of the thesis entitled **"Phytochemical Composition and Evaluation of the Antimicrobial, Antioxidant, Antibiofilm, and Antiquorum Sensing Activities of *A. polystachyus* Delile (Acanthaceae) Leaf Extracts.**" and developed by Meron Getahun Teferi 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

APPROVAL OF BOARD OF REVIEWER

We, the undersigned, members of the Board of Examiners of the proposal by Meron Getahun Teferi have read and evaluated the thesis entitled" **Phytochemical Composition and Evaluation of the Antimicrobial, Antioxidant, Antibiofilm, and Antiquorum Sensing Activities of *A. polystachyus* Delile (Acanthaceae) Leaf Extracts.**" 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 Master of Science in Applied Biology (Specialization in Biotechnology).

Chairperson	Signature	Date
Seid Mohammed (PhD)		02/08/2024
Internal Examiner	Signature	Date
Tura Safo (PhD)		02/08/2024
External Examiner	Signature	Date

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

Department-Head	Signature	Date
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Office of Postgraduate Studies, Dean	Signature	Date

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LIST OF ABBREVIATIONS AND ACRONYMS

AMR:	Antimicrobial resistance
AHL:	*N*-acylhomoserine lactone
AIP:	Auto inducing peptide
ANOVA:	Analysis of variance
AMPs:	Antimicrobial peptides
APHRRL:	Adama Public Health Research and Referral Laboratory Center
BST:	Brine shrimp lethality test
DPPH:	2, 2-diphenyl-1-picrylhydrazyl
eDNA	Extracellular DNA
EPS:	Extracellular polymeric substances
EO:	Essential oil;
FRAP:	Ferric reducing antioxidant power
GC-MS	Gas chromatography-mass spectroscopy
MBC:	Minimum bactericidal concentration
MBIC:	Minimum biofilm inhibitory concentration
MeOH:	Methanol extract
MDR:	Multi-drug resistance
MFC	Minimum fungicidal concentration
MHA:	Muller-Hinton Agar
MIC:	Minimum inhibitory concentration
MRSA:	Methicillin-resistant *S. aureus*
PDA:	Potato dextrose Agar
PIA:	Pyocyanin inhibition assay
PPL:	Pathogen priority list
ORAS:	Oxygen radical absorption capacity
RSA:	Radical scavenging activity
RI:	Relative inhibition
ROS:	Reactive oxygen species
ZOI:	Zone of inhibition

SUMMARY

The evolution of microbes in response to conventional antimicrobials leads to antimicrobial resistance (AMR) and multidrug resistance (MDR), which are global threats to public health. Natural products are possible solutions to this massive challenge. In this study, the phytochemical composition and antimicrobial and antioxidant potential of A. polystachyus extracts were investigated. Phytochemical extraction was done using maceration with methanol (99.9%). Phytochemical analysis was conducted using standard methods to test the presence of phenols, flavonoids, alkaloids, tannins, sterols and terpenoids, and glycosides. Chemical profiling of the leaf extracts and essential oil were performed using gas chromatography-mass spectrometry. *The anti-biofilm, antimicrobial, antioxidant, and antiquarian sensing activities were determined by using standard techniques. The result of the phytochemical components indicated the presence of alkaloids, flavonoids, phenols, sterols tannins, and terpenoids but the absence of glycosides in the extract. GC-MS analysis of methanol extract and essential oil detected 74 and 20 compounds, respectively. The major compounds identified in methanol extract and essential oil were beta-sitosterol acetate (16.06%) and Stearyl alcohol (25.38%) respectively. The antioxidant activity of the extract and essential oil ranged from 48.3 to 84.2 and 45.6 to 82 % RSA, respectively. The results of the antimicrobial activity of the extract revealed a high inhibition zone against Acinetobacter baumannii (12.3 ± 0.33), Pseudomonas aeruginosa (12.8 ± 0.33), and Staphylococcus aureus (18.6 ± 1.2), respectively. The MIC, MBC/MFC and MBIC values for extract were 0.5-1.0 mg/mL, 2-4 mg/mL, and 0.5-1.0 mg/mL; and for EO were 0.31-0.62 µL/mL,1.25-2.5 µL/mL, and 0.31-0.62 µL/mL, respectively, indicating inhibition potential as well as inhibition of biofilm formation. The tolerance test values indicated bactericidal activity against most strains and bacteriostatic/fungistatic activity against A. baumannii, E. faecalis, and C. albicans, respectively. The anti-quorum sensing activity of the extract achieved by Pyocyanin inhibition assay on P. aeruginosa showed 51.6% inhibition at 500 µg/mL. These results suggest that the extract and essential oil derived from A. polystachyus leaves are potent, valuable, cost-effective antioxidants and antimicrobials. Both extracts and their components may effectively combat pathogenic and resistant microbes.*

Keywords: *A. polystachyus, antimicrobial resistance, antioxidant, antibacterial, antifungal, biofilm, quorum sensing*

CHAPTER ONE

1. INTRODUCTION

1.1. Background and justification

In modern medicine, antimicrobial treatment is one of the main approaches, which is used to combat infectious diseases caused by pathogenic microorganisms (Hugo, *et al.*, 1998). However, the massive emergence and re-emergence of resistance against conventional antimicrobials pose a serious global threat to treating infections, which is a growing concern to human, animal, and environmental health (Walesch, *et al.*, 2023). In 2017, the World Health Organization (WHO) recognized antimicrobial resistance (AMR) as a major threat to global health in response to the massive increase in populations of multidrug-resistant strains (Jones, *et al.*, 2008; Bengtsson, *et al.*, 2018; WHO, 2017). The plausible causes of AMR include the excessive use of antibiotics against animals and humans, easy accessibility to antibiotics (over-the-counter), increased international travel, poor sanitation, and release of non-metabolized antibiotics or their residues into the environment through manure or feces (Jones, *et al.*, 2008). These factors contribute to genetic selection pressure for the emergence of multidrug resistance (MDR) microbes (Walesch, *et al.*, 2023).

MDR and AMR give rise to infections such as hospital-acquired, urinary tract infections (UTIs), ulcerative skin, lungs, ears, eyes, and catheters leading to increased medical costs, morbidity, and mortality. Several clinically relevant bacteria such as *E. coli*, *K. pneumonia*, *S. aureus*, *P. aeruginosa*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Acinetobacter* spp., *Enterobacter* spp., *Proteus* spp., and others cause infections, AMR, and MDR (De Oliveira, *et al.*, 2020; Mulani, *et al.*, 2019). *S. aureus* is considered the most notorious "superbug" (e.g., MRSA, CA-MRSA Community-associated methicillin-resistant *Staphylococcus aureus*). It is a nasal commensal of humans and can cause skin infections (DeLeo and Chambers, 2009). *A. baumannii* and *P. aeruginosa* are MDR opportunistic pathogens that may grow in niches with high antibiotic pressure where several other bacteria may not survive (Lye, *et al.*, 2012). AMR and MDR patterns in Gram-negative and Gram-positive bacteria give rise to infections that are either difficult to treat or impossible to cure with conventional antimicrobials (De Oliveira, *et al.*, 2020; Walesch, *et al.*, 2023).

A biofilm is a collection of microorganisms that adhere to a surface and create a matrix of extracellular macromolecular substances composed of microorganisms and extracellular polymeric substances (EPS) (Branda, *et al.*, 2005; Zhou, *et al.*, 2020). EPS are organic polymers involved in bacterial interaction with their environment and are mainly comprised of polysaccharides, proteins, extracellular DNA (eDNA), and lipids (Di Martino, 2018). Both G-positive and G-negative bacteria can form biofilms. The formation of biofilms occurs in two stages: the planktonic stage and the adherent stage. Biofilms are highly resistant to the host's immune system and antibacterial agents. Microbes inside biofilms can withstand 10-1000 times higher concentrations of antibiotics than cells in their plankton form (Høiby, *et al*., 2010). Antibiotic treatment can eliminate planktonic cells; however, it is difficult to treat biofilms (Khan, *et al*., 2020).

The increased dosage required to treat biofilm-forming microbes directly translates into an increased cost of treatment. The formation of biofilm and quorum sensing are the significant factors contributing to AMR and MDR (Branda, Vik, *et al*., 2005; Mukherjee and Bassler, 2019). Biofilm related to MDR significantly influences 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 (Magana, *et al*., 2018). Various mechanisms have been recognized for antimicrobial resistance by forming biofilms. Most notably, reduced permeability to antibiotics by the formation of a barrier, detoxification mechanism that produces enzymes to render the antibiotic inactive by disrupting or altering the antibiotic structure, reduction of intracellular concentration of antibiotics by drug efflux pumps, and drug sequestration by specific proteins that prevent the binding of antibiotics to their targets have been well documented (Branda, *et al.*, 2005; Simoes, 2011; Hamde, *et al*., 2022).

Quorum sensing (QS), an important cell-cell communication system, plays essential roles in regulating biofilm formation, virulence gene expression, drug efflux pumps, and plasmid transfer (Simoes, 2011; Mukherjee and Bassler, 2019). The communication between cells in QS-regulated systems was carried out by the production of QS signals in the form of diffusible auto-inducers (molecules), such as *N*-acyl Homoserine lactone (AHL) in Gram-negative bacteria and auto-inducing peptide (AIP) in Gram-positive bacteria (Mukherjee and Bassler, 2019). These signal molecules function via interaction with specific enzymes and receptor−activator proteins. For example, many Gram-negative bacteria use similar Lux I-type synthases and Lux R-type

activator proteins (Rutherford and Bassler, 2012; Deryabin, *et al.*, 2019). Therefore, developing antibiofilm agents and anti-QS inhibitors is crucial for developing antimicrobials; however, the difficulty in maintaining the pace of antibiotic discovery with the emerging and re-emerging resistant pathogens has mounted the current massive challenge to contain and treating infections (Rutherford and Bassler,2012; Mulani, *et al*., 2019). Therefore, there is an urgent need to search for and discover novel alternate treatment strategies to combat AMR and MDR. Several approaches have been developed including antibodies, vaccines, antimicrobial peptides (AMPs), probiotics, plant natural products, and Nano-biotechnology (Mengistu, *et al.*, 2023; Walesch, *et al*., 2023).

Natural products derived from plants and microbes have received particular attention (Atanasov, *et al*., 2021). The natural products in different parts of plants are comprised of alkaloids, essential oils (EOs), flavonoids, lectins, phenols, polypeptides, saponins, steroids, tannins, terpenoids, etc. These molecules are medicinally bioactive and exert antimicrobial action via different mechanisms that target various cellular processes. Flavonoids are complex with extracellular and soluble proteins and cell walls leading to antimicrobial activity (Hernández, *et al*., 2000). Fatty acid esters in EO possess antibacterial properties (Casillas-Vargas, *et al.*, 2021). Saponins are responsible for the leakage of proteins and enzymes from the cell (Zablotowicz, *et al.*, 1996). Steroids associate with membrane lipids and cause leakage from liposomes to show bactericidal action (Epand, *et al*., 2007), Tannins are involved in the inhibition of cell wall synthesis (Trentin, *et al.*, 2013). Terpenoids weaken the membranous tissue leading to the dissolution of the cell walls (Cowan, 1999), and several peptides are also known to be efficient antimicrobials (Atanasov, *etal*., 2021). Therefore, plant-derived natural products provide a wide array of compounds that may facilitate reducing and eradicating the load of pathogenic bacterial populations for treating infections (Atanasov, *et al.*, 2021).

EO have excellent therapeutic potential; however, the EO of this flowering plant species has yet to be explored. Therefore, in the present study, methanol extract (MeOH) and (EO) were prepared from the leaves of *A. polystachyus* Delile and investigated the qualitative (biochemical methods) and quantitative (gas chromatography-mass spectra (GC-MS)) phytochemical composition and evaluated the potential of the extracts for antioxidant, antibacterial, antibiofilm, and anti-quorum sensing properties. We expect that the results presented here will appreciate the medicinal potential of *A. polystachyus* Delile and contribute toward combating pathogenic and

resistant bacteria responsible for causing fatal diseases. The GC-MS analysis is a valuable method that has increasingly been applied to medicinal plants for nonpolar components, volatile EO, fatty acids, and alkaloids. GC-MS is a helpful technique for the compositional analysis of phytochemicals in extracts.

A. polystachyus Delile is a shrub belonging to the family *Acanthaceae* and native to Burundi, Ethiopia, Kenya, Rwanda, Sudan, Tanzania, and Uganda. This species grows at medium to high altitudes (1000-3200 m) in different parts of Ethiopia and is characterized by pink flowers and soft, hairy leaves (Kifle and Atnafie, 2020). The *Acanthaceae* family is pharmacologically important for antifungal, anti-inflammatory, antipyretic, antioxidant, antiviral, antimalarial, insecticidal, hepatoprotective, immunomodulatory, and antiplatelet activities (Okoli, *et al.*, 2008; Olatunji, *et al*., 2022). *A. polystachyus* Delile has been investigated for antimalarial and wound healing activities (Demilew, *et al*., 2018; Kifle and Atnafie 2020); however, there is only one report regarding its antimicrobial potential (Mailu, *et al.*, 2021). To the best of our knowledge, the inhibition of biofilm formation and quorum sensing by this plant species has been investigated. Furthermore, a detailed analysis of the composition of extracts of this important medicinal plant has yet to be elucidated. The knowledge and understanding of the constituents of extracts are essential to elucidating the mechanisms of action. In most reported studies, solvents such as methanol, water, and acetone have been used for extraction from leaves and roots.

1.2. Statement of the Problem

Among many underdeveloped countries today, microbes are thought to be the primary cause of disease and mortality, particularly in humans. *E. faecalis, S. aureus, S. pyogenes, A. baumannii, P. aeruginosa, and E. coli,* are the most common bacterial pathogens. Despite the discovery of new antibiotic kinds in recent years, the prevalence of antibiotic resistance among many bacterial diseases has significantly increased. According to the WHO report, bacteria–antibacterial drug combinations resistance for *E. coli*/3rd generation cephalosporin, *E. coli*/ fluoroquinolones, and Methicillin-resistant *S. aureus* (MRSA) is 85, 90, and 86, respectively. High rates of MRSA imply that treatment for suspected or verified severe *S. aureus* infections, such as common skin and wound infections must rely on second-line drugs in many countries, and that standard prophylaxis with first-line drugs for orthopedic and other surgical procedures

will have limited effect in many settings. Second-line drugs for *S. aureus* are more expensive; also, they have severe side effects for which monitoring during treatment is advisable, increasing costs even further. High proportions of resistance to 3rd generation cephalosporins reported for *E. coli;* means that treatment of severe infections likely to be caused by these bacteria in many settings must rely on carbapenems, the last resort to treat severe community and hospital-acquired infections. These antibacterial are more expensive, may not be available in resource-constrained settings, and are also likely to further accelerate the development of resistance (Organization, 2014).

The global problem of antimicrobial resistance is particularly pressing in developing countries, where the infectious disease burden is high and cost constraints prevent the widespread application of newer, more expensive agents (Okeke, *et al.,* 2005). The antibacterial activity is a prominent quality of these extracts. Continual development of bacterial resistance toward various antibiotics can cause grave and potentially life-threatening infections by Gram-positive and Gram-negative bacteria and has stimulated the development of new antimicrobial drugs to treat these types of bacterial resistance (Sathya & Shoba, 2014). In addition to antibiotic resistance, prolonged antibiotic use may kill bacteria beneficial to health. Excessive use of antibiotics may disturb the intestinal microflora and lead to the proliferation of pathogenic bacteria such as *Clostridium* (Ofosu, 2016).

Although *A. polystachyus* has been demonstrated as one of the medicinal plants widely dispersed in different parts of Ethiopia and has been traditionally used in the treatment of different diseases, there is a lack of sufficient studies regarding its antimicrobial, antioxidant, and antibiofilm activity for combating antimicrobial resistance (AMR).

1.3. Objective of the Study

1.3.1. General Objective

- ✓ To investigate and analyze various phytochemicals from *A. polystachyus* and leaf extracts and evaluate their antibacterial, antioxidant, and antibiofilm activities against selected pathogenic antibiotic-resistant bacterial species.

1.3.2. Specific Objectives

- ✓ Analyze the phytochemical composition of the leaf extract of *A. polystachyus*.
- ✓ Analyze the EO composition of leaf extract of *A. polystachyus*
- ✓ Evaluate the antibacterial activity of the crude methanol leaf extract of *A. polystachyus* Delile. against Gram-positive and Gram-negative human pathogens
- ✓ Determine Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of methanol leaf extract of *A. polystachyus* Delile.
- ✓ Evaluate the antioxidant activity of the methanol leaf extract of *A. polystachyus* Delile. Using DPPH assays.
- ✓ Evaluate the antibiofilm activity of the methanol leaf extract of *A. polystachyus* Delile by Micro titer plate biofilm assay.
- ✓ Evaluate the inhibition of Quorum sensing (QS) of methanol leaf extract of *A. polystachyus* Delile.

1.4. The Scope of the Study

The study was conducted at Adama Science and Technology University. It was concentrated on the phytochemical screening of *A. polystachyus*, as well as the antioxidant and antimicrobial activities of the pathogenic bacteria species, which are responsible for various human diseases. Many bacterial species today are resistant to drugs. The antibiofilm impact of phytochemical compounds, however, makes it possible for bioactive compounds extracted from medicinal plants to suppress resistant microbial species.

1.5. Significance of the Study

This research is expected to provide information and some knowledge about *A. polystachyus* by extracting various phytochemicals from the leaf part of this plant as well as provide its effectiveness in antioxidants. Provide scientific support to traditional uses of the plants to treat infectious diseases like antibacterial, antibiofilm, and Quorum sensing (QS) activities.

The other significance that this research expected is to give a scientific explanation for traditional use claims of this medicinal plant in Ethiopia. Moreover, the study provides baseline information for future pharmacological studies and the researchers who are interested in conducting further work on the area.

CHAPTER TWO

2. LITERATURE REVIEW

2.1. Medicinal plants

Medicinal plants are any plants that, in one or more of their organs contain substances that are used for therapeutic purposes or precursors of the synthesizing of valuable drugs. These substances are important in preventing and treating various ailments and diseases, which are considered beneficial in the health care system. They contain promising molecules used by the population of the world to meet their primary healthcare system (Nigussie, *et al.*, 2021). These natural compounds as known as secondary metabolites (phytochemicals) have no direct contribution to their growth, development, and metabolism (Equar, *et al*., 2018). Ruther then play a role play in defense against plant enemies (biotic and abiotic stresses), and pollution, provides the ability to survive stress and UV rays, and also contributes to the color, flavor, and aroma concerning the plan (Balamurugan, *et al.,* 2019). They are classified into major groups including phenols (45%), terpenoids (10%) steroids (27%), and alkaloids (18%) (Saxena, *et al.*, 2013).

Plant-derived natural compounds or bioactive compounds have a paramount role in mitigating oxidative stress due to their antioxidant activity (Ahmed, *et al*., 2019; Mahmood, *et al*., 2019). Because various crude plant extracts have a high oxidative capacity and high concentration of phenol compounds (Dessalegn, 2020). In our body, various biochemical reactions can generate an unbalanced quantity of free radicals that results in oxidative damage of lipids, proteins, and other important biomolecules by creating abnormal physiological conditions. This oxidative damage was responsible for the development of several illnesses such as cancers, diabetes, cardiovascular disorders, neurodegeneration, aging, and others (Khanal, *et al.*, 2020). Natural antioxidants have an indispensable role in repairing the damage caused by the reactive oxygen species (ROS) by converting free radicals into harmful molecules by radical chain reaction (Wang, *et al.,* 2013).

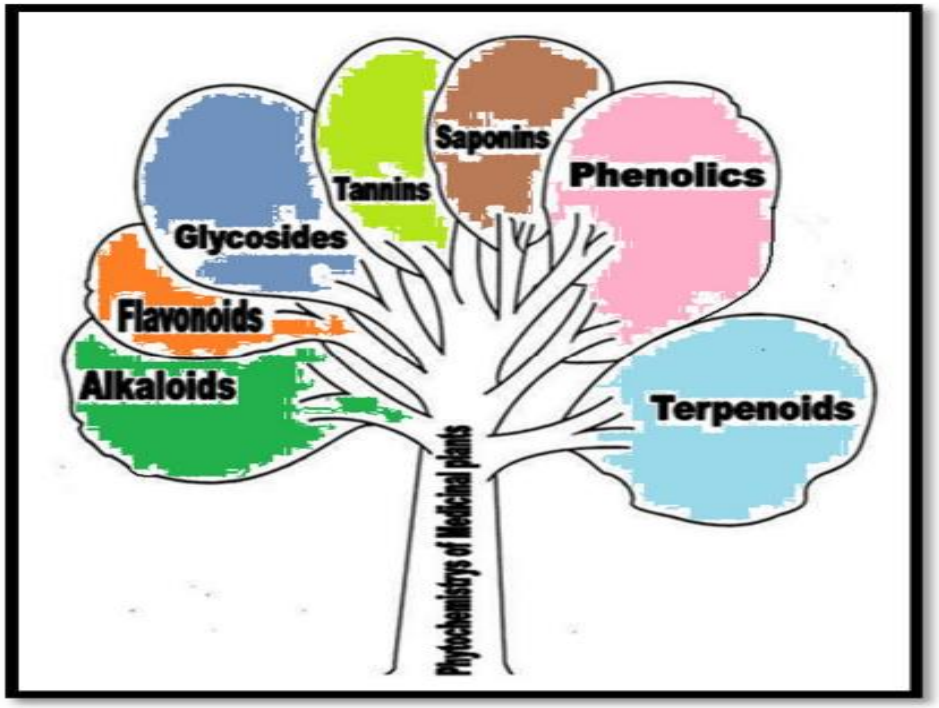


Figure 1: Photochemistry of medicinal plants (Saxena, *et al.*, 2013).

These compounds possess different biological properties within plants that contribute to treating a wide spectrum of diseases including cancers, neurological disorders, chronic inflammation, and lesions, particularly due to diabetes, cardiovascular diseases, and wounds (Nigussie, *et al.*, 2021). Medicinal plants have a vital role in antimicrobial activities. Medicinal plants have in-vitro antimicrobial activities or properties because of rich in various secondary metabolites, such as terpenoids, tannins, flavonoids, and alkaloids (Górniakh, *et al*., 2019).

2.2. Phytochemicals

Phytochemicals are bioactive compounds that are naturally active occurring in plants naturally occurring in plants and provide health benefits for human beings. They protect plants from damage or several environmental hazards and diseases and provide color, flavor, and aroma. More than 4500 phytochemical compounds have been identified and 350 of them are studied in detail (Koche, *et al*., 2016). They are divided into classes based on their functional protective role, physical, and chemical characteristics (Saranya & Divyabharathi, 2019). Different major classes of these compounds are described as follows:

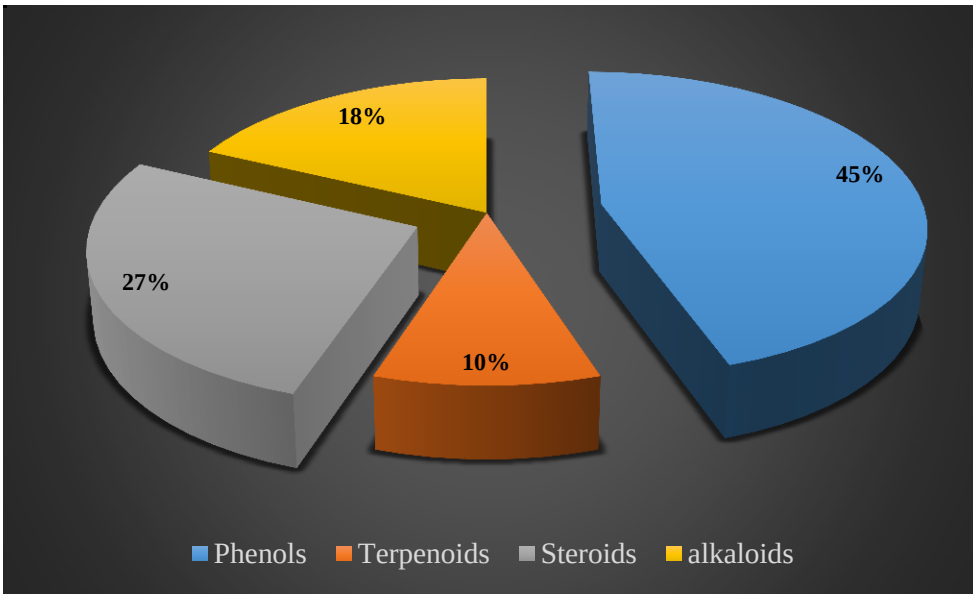
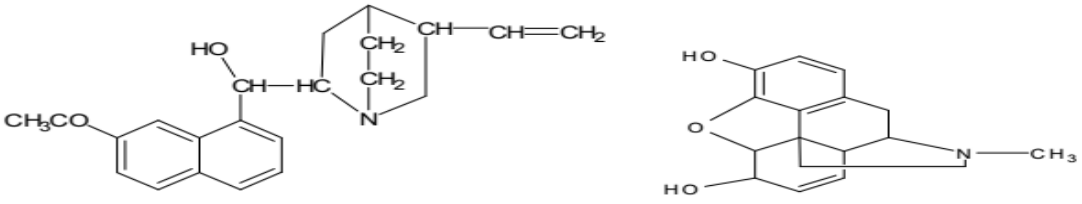


Figure 2: A major component of secondary metabolite found in the plant leaves.

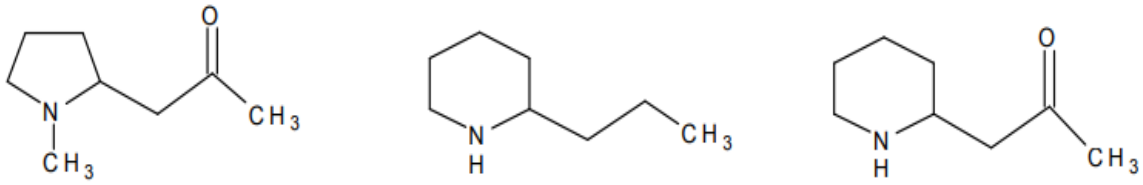
2.2.1. Alkaloids

Alkaloids are a natural product and their name is derived from alkaline which describes a nitrogen-containing base. These compounds are naturally synthesized by animals, bacteria, fungi, and plants. However, alkaloids are primarily found in plants, especially in flowering plants, and metabolite by-products that are derived from amino acids (Naseem, *et al*., 2014). In the early 19th century among natural bioactive compounds isolated from medicinal plants, alkaloids were the dominant compound and were known as vegetable alkalis with a bitter taste (Saxena, *et al*., 2013).

Alkaloids are used as local anesthetics and stimulants such as cocaine (Pang, *et al*., 2012). Which have numerous biological and pharmacological activities including antimicrobial, insecticidal, antimalarial, antihypertensive, antiarrhythmic effects, and anticancer action (Saxena, *et al*., 2013; Wink, *et al*., 2004). Many alkaloids also have stimulant properties due to caffeine and nicotine. Quinine and morphine are used as analgesic and antimalarial drugs respectively (Pang, *et al*., 2012).



Quinine Morphine

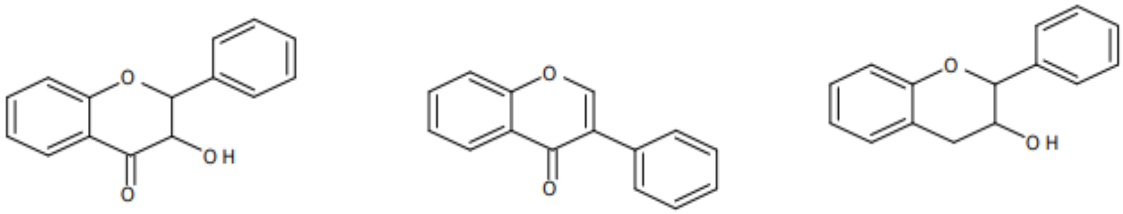


Hygrine Coniine Isopelletierine

Figure 3*:* Structures of some important alkaloids (Naseem, *et al.*, 2014).

2.2.2. Flavonoids

Flavonoids are also known as bioflavonoids polyphenolic antioxidant compounds that exist everywhere in nature. The majority of them identified as vegetables, fruits, tea, coffee, and fruit drinks. They have been present as methylated derivatives, aglycones, and glucosides (Atmani, *et al*., 2009). Flavonoids have obtained various attention from scientists because of their wide biological and pharmacological activities. Scientists are interested in flavonoids due to their broad range of biological and pharmacological properties, such as anti-inflammatory, antimicrobial, antitumor activities, cytotoxicity, enzyme inhibition, vascular activity, etc. (Lin & Weng, 2006). Nonetheless, the antioxidant activity is one of the most significant and potent characteristics of various families of flavonoids. These compounds' capacity for a kind of action depends on their molecular makeup, where the hydroxyl groups are located, and other attributed features in chemical structures (Tapas AR, *et al*., 2008).



Flavanonol Isoflavone Flavan-3-ols

Figure 4*:* Representative Chemical structures of some flavonoids (Atmani, *et al.*, 2009).

2.2.3. Glycosides

Glycosides are the condensation product of sugars that chemically contain carbohydrates (glucose) and non-carbohydrates (aglycon or genin) that exist within the cell cap. They are

classified into diverse classes based on the linkage between sugar (glycogen) and aglycon moieties like oxygen, carbon, sulfur, or nitrogen and also based on the chemical nature of aglycon molecules such as alcohol, aldehyde, or steroids. These phytochemicals are essential for the treatment of various illnesses by acting on gustatory. For instance, anthracene glycosides are used for the treatment of skin diseases and chalcone glycosides have an anticancer activity or anticancer agents (Hartwig, *et al*., 2012; Pereira, *et al.*, 2009).

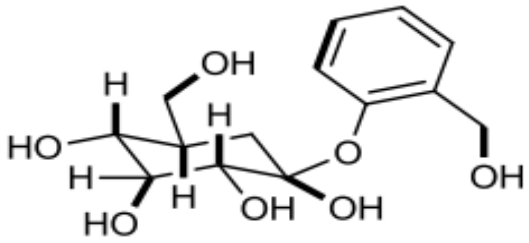
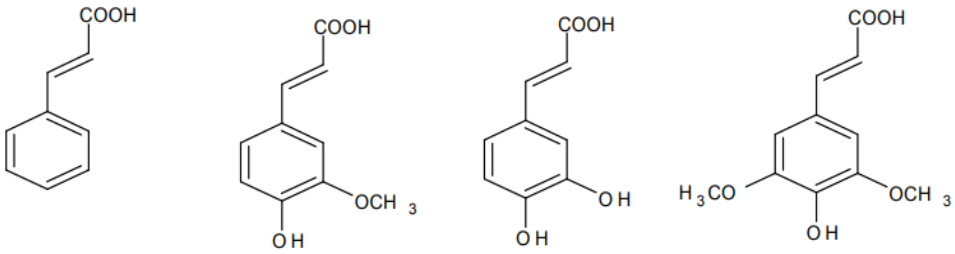


Figure 5: Structure of glycosides (Hartwig, *et al.*, 2012; Pereira, *et al*., 2009).

2.2.4. Phenolics

Phenols are the largest group of secondary metabolites that widely exist in plants. They are a vast and varied class of the plant's chemical components (Walton, *et al.*, 2003). Phenols are chemical compounds with an aromatic hydrocarbon group attached to a hydroxyl group (-OH). There are three most important groups of dietary phenolics include flavonoids, phenolic acids, and polyphenols. Phenolics are used as a defensive compound and have several qualities that help people by protecting them from different diseases. Their antioxidant qualities are especially important in avoiding diseases that are caused by free radicals (Saxena, *et al*., 2013).



Cinnamic acid Ferulic acid Sinapic acid Caffeic acid

Figure 6: Phenolics Chemical structures (Saxena, *et al.*, 2013).

2.2.5. Saponins

Saponins are secondary metabolites widely distributed in plants. They are an important group of glycosides which are containing one or more sugar chains on a triterpene or steroid aglycone backbone also called a sapogenin. They form stable foam such as soap in an aqueous solution and their name is derived from the Latin word 'SOPA', which means the plant consisting of the frothing agent when diluted in an aqueous solution (Saxena, *et al.*, 2013; Naseem, *et al.*, 2014). These include various compounds with structural diversity which are reflected in their biological and physiochemical properties such as contributing to the number of traditional practices such as soaps, fish poison, pes, and molluscicides as well as industrial applications. In addition, plant extract containing saponins has served as surface active, detergents, and foaming agents in the industry and is widely used in food. However, traditionally considered anti-nutritional and in some cases limited their use due to their bitter taste (Troisi, *et al*., 2015). Moreover, these compounds also have several health benefits because their structural variety acts as an antioxidant to impair protein digestion and uptake of the vitamins and minerals in the gut, also as antiviral and fungal (Shi, *et al*., 2004; Juang & Liang, 2020).

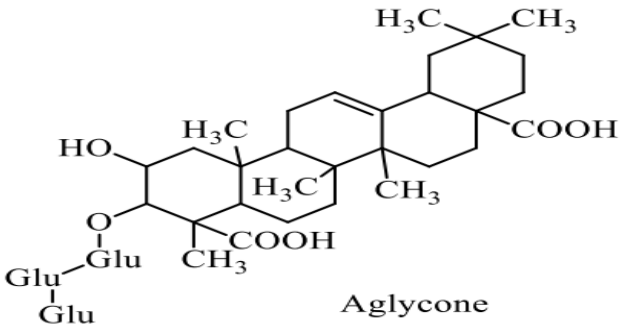


Figure 7: General structure of Saponins (Troisi, *et al.*, 2015).

2.2.6. Steroids

Steroids are secondary metabolites with diverse structures and biological functions. They are organic compounds with four cyclohexane rings and are derived from cholesterol. Various group of synthetic steroids plays a crucial role in medicinal value through their use as contraceptive drugs (Lopez, *et al.*, 2014), cardiovascular agents (Rattanasopa, *et al*., 2015), antibiotics, anesthetics, and anti-inflammatory (Aav, *et al*., 2005).In addition, steroid

compounds have been used to reduce stress, reduce cholesterol levels, activate the immune system, treat tumor cells in cancer cases, and improve memory and learning (Visweswari, *et al*., 2013).

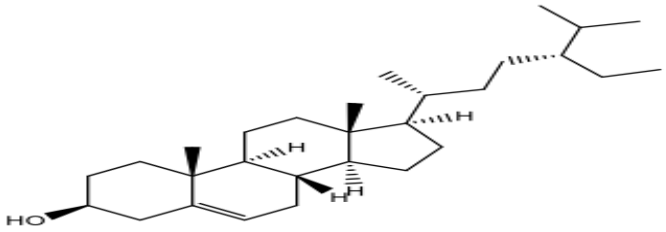
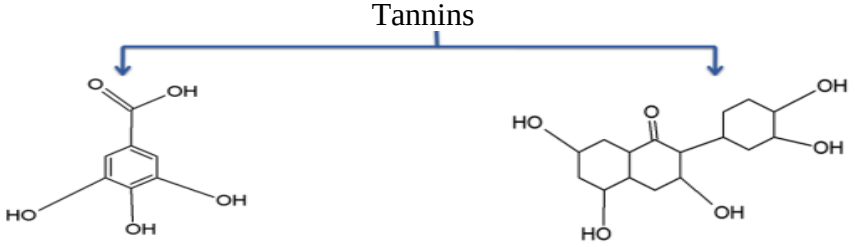


Figure 8: Structure of some naturally occurring steroids (Rattanasopa, *et al.*, 2015).

2.2.7. Tannins

Tannins are toxins that have the potential to reduce the growth and survivorship of many herbivores when added to their diets. They also serve as a repellent to feeding a wide variety of animals. Additionally, due to interaction with salivary protein, they can enhance unpleasant, harsh, and astringent sensations in the mouth of the human and can activate herbivores' digestive enzymes by binding with protein (Chappell & Hahlbrock, 1984). They are divided into two main categories: namely hydrolysable tannins and condensed tannins. Hydrolysable tannins are heterogeneous polymers containing phenolic acids, especially simple sugars, and gallic acid.

The condensed one is formed by the linkage of flavonoid units and common constituents of woody plants that can be hydrolyzed to anthocyanidins through treatment with strong acids. Due to this referred to as proanthocyanidins. Tannins are secondary metabolites compounds that have shown various biological activities such as anti-inflammatory, antimicrobial, and antiseptic activity, against diarrhea and stomachache (De Bruyne, *et al.*, 1999; Dolara. *et al*., 2005). They are employed in several industries such as in the dyestuff industry as caustics for cationic dyes and production of drink and food industry to clarify wine, beer, and fruit juices.

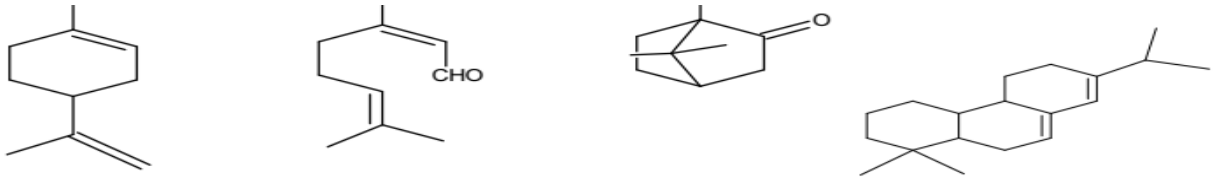


Hydrolysable Tannins Condensed Tannins

Figure 9: Types and the basic structure of Tannins (Dolara. *et al.,* 2005).

2.2.8. Terpenoids

Terpenoids are a class of natural products that have been derived from five-carbon isoprene units and classified into several classes according to the number of isoprene units (Langenheim, 1994). Terpenes are boundless and mainly or especially distributed in plants as constituents of essential oil. These natural products have several commercial applications such as flavor and fragrances in foods and cosmetics. Also important for the quality of agricultural products, served as the flavor of fruits and fragrances of the flowers like linalool. Various plants produce volatile terpenes to attract pollinators such as insects. As several preliminary investigations have shown they play a vital role as signal compounds and growth regulators of plants. Terpenoids have exhibited potential medicinal properties, such as antibacterial, anti-cancer activities, anticarcinogenic, anti-inflammatory, antimalarial, anti-ulcer, antiviral, hepaticidal, and inhibition of cholesterol synthesis, (Dudareva, *et al*., 2004; Langenheim, 1994; Mahato & Sen, 1997).



Limonine Citral Camphor Abietic acid

Figure 10: Structures of some Terpenoids (Dudareva, *et al.,* 2004).

2.3. Botanical description of Acanthaceae Family

Acanthaceae also referred to as the *Acanthus* family, is a family of dicotyledonous flowering plants in the *Lamiales* order that includes almost 250 genera and about 4000 species, the majority of which are tropical herbs, shrubs, twining vines, and epiphytes. Only a few species are found in temperature regions, with the majority of them being found in Indonesia, Malaysia, Africa, Brazil, and Central America. Typically, leaves are opposite and stipule-free. Flowers are bisexual, zygomorphic to sub-actinomorphic, and typically arranged in racemes or panicles with terminal or axillary (Chang, 2017). Some significant members of this family of plants are used to treat edema, pneumonia, antidiarrheal, malaria, wounds, cough, and eye infections (Khan, *et*

al., 2017). The researchers were attracted to investigate the many genera and species of this family because of its medical significance. Antifungal, anti-inflammatory, antioxidant, anti-platelet, antipyretic, antiviral, cytotoxic, hepatoprotective, insecticidal, and immunomodulatory aggregation properties are all present in the Acanthaceae family (Assefa, *et al.*, 2016). Acanthaceae family plants phytochemical examination revealed the presence of several phytochemicals including benzenoids, flavonoids, glycosides, naphthoquinone, phenolic, and triterpenoid compounds. These substances contribute significantly to numerous biological processes and fight a variety of fatal diseases (Awan, *et al.*, 2014).

2.3.1. Genus *Acanthus*

The bulk of the species belonging to the *Acanthus* genus, which is a part of the Acanthaceae family, are found in tropical and subtropical climates on all seven continents. The name *Acanthus* comes from the Greek word "*Acanthus*," which describes a thorn or thistle and refers to some species' thorny leaves. Typically, the opposing leaves of these shrubby or herbaceous plants lack stipules and are arranged in a basilar rosette. They typically feature a bilabial or unilabiate corolla and two or four epipetalous stamens (Matos *et al.*, 2022). *Acanthus* is a genus of flowering plants belonging to the family Acanthaceae. The *Acanthus* family is large with some 2500-3000 species in about 250 genera. The family is distributed in subtropical and tropical habitats mainly around Africa, Brazil, Central America, and Indo-Malaysia. The Acanthaceae family possesses antifungal, anti-inflammatory, antioxidant, antipyretic, antiviral, cytotoxic, insecticidal, hepatoprotective, immunomodulatory, and anti-platelet aggregation activities (Assefa, *et al.*, 2016).

2.3.2. *A. polystachyus* Delile

A. polystachyus Delile is a shrub or small tree up to 7 meters which is ecologically widespread and locally cultivated from medium to high altitudes (1000-3200m) in Moist and Wet Kolla, Weyna Dega and Moist Dega agro-climatic zones of Ethiopia (Raina, *et al.*, 2008). This species has pink flowers, and soft, hairy leaves and grows slightly larger. *A. polystachyus* Delile *is* native to Burundi, Ethiopia, Kenya, Rwanda, Sudan, Uganda, and Tanzania.

A. puberscens (Oliv) Engl is used in traditional medicine for the treatment of gonorrhea and syphilis in Tanzania (Moshi, *et al.*, 2010). The root extracts of *A. puberscens* (Oliv) Engl

exhibited weak antibacterial and antifungal activity. Using the brine shrimp's lethality test ethanol and aqueous extracts were virtually non-toxic to brine shrimp larvae, but the dichloromethane extract exhibited mildly toxic effects. The brine shrimp lethality test (BST) was used to predict the presence of cytotoxic activity in the extracts. An 80% ethanol extract from the leaves exhibited antifungal activity a weak antibacterial activity and also antiviral activity against the poliovirus and measles viruses (Vlietinck, *et al.,* 1995). The root extracts of *A. puberscens* exhibited weak antibacterial activity against Gram-positive (*S. aureus, S. agalactiae,* and *S. faecalis*) and Gram-negative bacteria (*S. typhi* and *P. aeruginosa*) and antifungal activity against *Candida albicans*. The roots are used for the treatment of gonorrhea and syphilis. The decoction of the leaves is used for the treatment of anthrax, gastroenteritis, and pneumonia. It is also reported that preparation of the dried leaves is used externally as a remedy for scabies in Rwanda (Moshi, *et al.,* 2010).

In Ethiopia, *A. polystachyus* Delile is traditionally used for treating scorpion stings with root decoction given orally and to treat bleeding and stabbing pain leaf paste is applied. The leaves are used as a medicine with butter and applied to wounds (Teklehaymanot, *et al.,* 2007). The leaves are powdered and mixed with butter pasted on the wound and exposed to sunlight for a few minutes to treat wounds. The root is crushed, squeezed with water & taken orally at night to treat trachoma (Bussmann, *et al.,* 2011; Gebeyehu, 2016; Teklehaymanot, *et al.,* 2007). The root is also used for treating malaria and intestinal worms. The roots are powdered and mixed with cold water are given orally to the Dog as a vaccine (Chekole, *et al.,* 2015; Teklehaymanot, *et al.,* 2007).

Table 1: Taxonomical Classification of *A. Polystachyus* Delile.

Kingdom	**Plantae**
Phylum	Tracheophyte
Class	Magnoliopsid
Order	Lamiales
Family	*Acanthaceae*

Genus	*Acanthus*
Species	*Polystachyus*

2.3.3. Ethnobotanical Uses of *Acanthus* species

Only a few species in the genus *Acanthus* have been described based on research on traditional uses. Only nine of the 29 species recognized by World Flora Online were reported for their usual uses: *A. arboreus, A. hirsutus, A. ilicifolius, A. leucostachyus, A. mollis, A. montanus, A. spinosus, and A. ebracteatus* are among the species (Matos *et al.*, 2022). Some common species in this genus are well known for their therapeutic properties all over the world, as seen in the table below.

Table 2: Some medicinal plants of Acanthus species and parts of use.

Species	Traditional uses	Parts of used	Ways preparation	Reference
A. arboreus	For snake bite	NM	Applied on effect area	(Ameret, *et al.*, 2004)
A. ebracteatus	Treating conditions associated with inflammation	Whole parts plant	Decoction	(Hokputsa, *et al.,* 2004)
A. eminens	For backache, cough, eye infections, wounds, antidiarrhea, and edema.	leaves	Infusions	(Woldemariam, *et al.*, 2016)
A. hirstu	as an expectorant, for wound healing, and constipation	Stem and leaf	NM	(Uysal, *et al.*, 2018)

A. ilicifolius	For asthma, diabetes, hepatitis, ringworm, rheumatism	Leaves	Decoction and soaking in water for external	(Saranya, *et al.*, 2015)
A. leucostachyus	Treating toothache, fever, small-scale cuts, burns, and wounds	leaves	Pest	(Dev, *et al.*, 2022)
A. Mollis	To treat wounds, psoriasis, abrasions, and swollen legs	Leave root	Applied as poultice, gargle, and decoction	(Adeyemi, *et al.*, 2005)
A. polystachyus Delile	Treat malaria, cough wounds heal	leave	Decoction	(Demilew, *et al.*, 2018)
A. sennii	Treating scorpion sting	root	Decoction	(Assefa, *et al.*, 2016)

NM: not mentioned

2.4. Pharmacological study of *Acanthus* species

The principal ailments treated with *Acanthus* species are respiratory, neurological, and reproductive system disorders, and gastrointestinal, urinary, and skin conditions. The three species that are most commonly utilized are *A. ebracteatus*, *A. ilicifolius*, and *A. montanus* (Matos, *et al.*, 2022). Among the species of this genus, *A. ilicifolius* has undergone substantial pharmacological research. According to the study, *A. ilicifolius* exhibits considerable bioactivities including antibacterial, anti-inflammatory, anti-leishmanial, anti-nociceptive activity, anti-ulcer, and osteoblastic effects (Firdaus, *et al.*, 2013; Islam, *et al.*, 2012). It has been stated that several portions of this plant have anti-inflammatory and anti-osteoporotic properties (Van, *et al.*, 2008). Due to the presence of benzoxazolinone, *A. ilicifolius* is utilized as an anticonvulsant, hypnotic, and skeletal muscle relaxant; an alkaloid with CNS depressive effect has also been observed (Amer, *et al.*, 2004). *A. montanus* leaves are believed to have

antibacterial and immunologic properties, as well as anti-inflammatory, anthelmintic, and antipyretic properties. Additionally, it exerts peripheral analgesic and antipyretic effects on both short-term and long-term inflammatory processes (Asongalem, *et al.*, 2004). *Acanthus sennii* has antifungal, anti-inflammatory, antioxidant, antipyretic, antiviral, cytotoxic, hepatoprotective, and immunomodulatory, insecticidal, properties (Assefa, *et al.,* 2016). Analgesic, antibacterial, anticancer, anti-feedant, antifertility, anti-hepatotoxic, anti-inflammatory, antioxidant, and antiviral, activities have been discovered in several *Acanthus* species (Çapanlar, *et al.,* 2010). There were discovered to be Aliphatic alcohol, alkaloids, benzoxazinoids, flavonoids, glycosides, lignans, megastigmanes, phenylethanoids, and saponins among this genus' constituents (Kanchanapoom, *et al.,* 2006). The alcohol-extracted root a*nd* leaves of *Acanthus ilicifolius* showed high inhibitory effects against *Bacillus subtilis, Staphylococcus aureus, Candida albicans, Aspergillus fumigatus, and Aspergillus Niger,* and moderate inhibitory effects against *Pseudomonas aeruginosa and Proteus Vulgaris* (*Bose & Bose*, 2008).

Table 3: Bioactive Phytochemicals in Medicinal Plants.

Main groups of compounds	Classification	Biological function
Cellulose, hemicellulose, gums, mucilage's, pectin's, lignin's	NSA (non-starch polysaccharides.)	Water holding capacity, delay in nutrient absorption, binding toxins and bile acids
Terpenoids, alkaloids, phenolics	Antibacterial & Antifungal	Inhibitors of micro-organisms, reduce the risk of fungal infection
Polyphenolic compounds, flavonoids, carotenoids, tocopherols, ascorbic acid	Antioxidants	Oxygen free radical quenching, inhibition of lipid peroxidation
Carotenoids, polyphenols, curcumine, Flavonoids	Anticancer	Inhibitors of tumor, inhibited development of lung cancer, anti-metastatic activity

Reductive acids, tocopherols, phenols, indoles, aromatic isothiocyanates, coumarins, flavones, carotenoids, retinoids, cyanates, phytosterols	Detoxifying Agents	Inhibitors of procarcinogen activation, inducers of drug binding of carcinogens, inhibitors of tumorigenesis
Alkaloids, terpenoids, volatile flavor compounds, biogenic amines	Other	Neuropharmacological agents, anti-oxidants, cancer chemoprevention

Researchers are now devoted to the progress of innocent or harmless biologically active plant-derived compounds to employ for the development of novel drugs (Gaire & Subedi, 2011). The involvement of these drugs in medical practice has shown that they are relatively non-toxic, safe, and even free from serious side effects (Górniak, *et a*l., 2019). As a result, various scientific studies are attracted to the antimicrobial and antioxidant activities of plant extracts in order to explore an alternative therapy against different types of a pathogen (microbes) and oxidative reactions (Marasini, *et al*., 2015).

2.5. Antioxidant

An antioxidant is a molecule capable of inhibiting the oxidation of other molecules. On the other hand, an antioxidant is defined as a substance that directly scavenges ROS or indirectly acts to up-regulate antioxidant defenses. In terms of food, an antioxidant is any substance that presents in low concentration when compared to that of an oxidizable substrate and is significantly able to inhibit the oxidation of that substrate. However, later defined as any substance that prevents or removes oxidative damage to a target molecule (Halliwell, *et al*,. 1995).

An antioxidant is the constituent of plant materials that act as radical scavengers and contribute to converting radicals to less reactive species. Various types of antioxidants are found in dietary sources like vegetables, fruit, and tea (Mandal, *et al.,* 2009). This plays a crucial role both in the food system and the human body to mitigate oxidative processes and the harmful effect of ROS (Çakmakçi, *et al*., 2015). Antioxidant compounds can improve shelf-life by retarding the process of lipid peroxidation and scavenging or inhibiting free radicals which are the major reasons for

the deterioration of food and pharmaceutical products at the time of processing and storage (Halliwell, 1996). In the food system, the involvement of nutritional antioxidant molecules is also able to prevent the formation of secondary lipid peroxidation products by maintaining the color, aroma, and texture of the food product during storage (Çakmakçi, *et al*., 2015).

Furthermore, antioxidants also can protect the human body from free radicals and ROS effects. They delay the progression of chronic diseases, cancer, and age-related diseases such as coronary heart disease and prevent the radical chain reactions of oxidation when added to food (Gulcin, 2020). Phenols and flavonoids are the major bioactive compounds of these natural sources which are responsible for their health benefits.

Antioxidant capacity is the overall ability of an organism or food to catch free radicals and prevent their harmful effects. One of the most regularly detected biological activities in plant extracts is antioxidant behavior. In order to determine the antioxidant capabilities of various plant parts and their derivatives, a variety of tests have been routinely used (Bondet, *et al.*, 1997). These are DPPH (2, 2 diphenyl-1-picrylhydrazy), FRAP (ferric reducing antioxidant power), ORAS (oxygen radical absorption capacity), and ABTS. Among them, DPPH is discussed below.

2.5.1. DPPH free radical scavenging antioxidant activity

2, 2-diphenyl-1-picrylhydrazyl is a stable free radical with a purple color, which shows hydrogen acceptor ability towards antioxidants. Hence, it is commonly used in DPPH assay for measuring the antioxidant activity of different natural samples such as wine, fruits, herbal tea, etc. It involves the use of free radical, DPPH which is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors and to evaluate the antioxidant activity (Granato, *et al*., 2018; Sudhakar, *et al*., 2018). The reduced DPPH-H is formed when a free radical scavenger pairs the odd DPPH radical electron with hydrogen. Up on reacting with antioxidants, produces corresponding hydrazine DPPH-H and it becomes a pale yellow color (Bondet, *et al*., 1997). A strong absorption spectrum is caused by the DPPH free radical's odd electron at 517 nm. DPPH assay has various advantages over other assays, like simple, rapid, inexpensive, antioxidant of a complex biological system can be quantified, assay results are reproducible and have better sensitivity than other radical scavenging methods, multiple samples

can be screened and thermally unstable compound can be measured efficiently. Since radical scavenging is measured at room temperature. The below scheme shows DPPH free radical accepts hydrogen from antioxidant compounds.

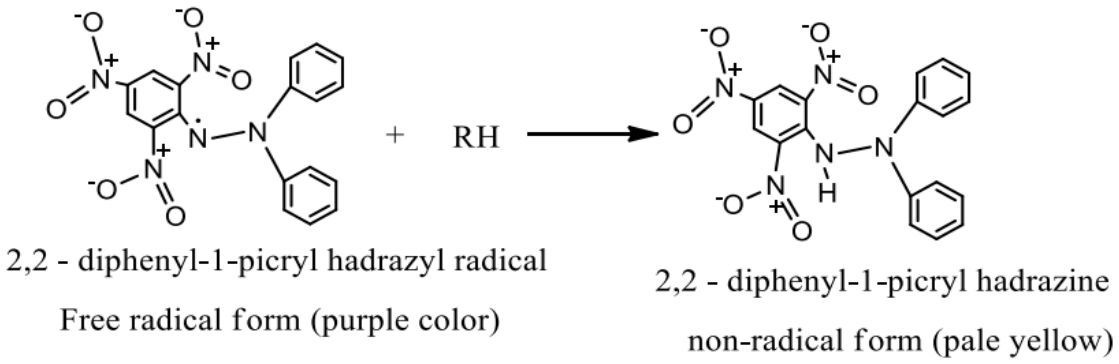


Figure 11: Reaction of DPPH with Antioxidants

2.6. Biofilm

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). In most biofilms, microorganisms make up less than 10% of the dry matter, while matrix can make up to 90% or more. EPS is primarily composed of polysaccharides, proteins, extracellular DNA (e DNA), and lipids, most of which are produced by the organism itself the stability of the matrix is ensured by non-covalent bonding between EPS (Di Martino, 2018). The architecture of biofilms is influenced by many factors, including hydrodynamic conditions, concentration of nutrients, bacterial motility, and intercellular communication as well as exopolysaccharides and proteins. Various natural antibiofilm agents including phytochemicals, bio-surfactants, antimicrobial peptides, and microbial enzymes have different mechanisms of action against interfering with biofilm formation *(*Mishra, *et al.*, 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 (hospital-acquired infection or a health-care associated infection) 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 Predominant bacterial species that cause MDR including *P. aeruginosa, S. aureus, E. coli, S. epidermidis, and S. viridans* has also been found to coexist with *S. aureus* in the lungs of cystic fibrosis patients and on wounds (Abraham, *et al.*, 2012).

2.6.1. 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 (Zhou, & Cai, 2018). The developmental stages leading to biofilm formation appear to be conserved but, every species forms a unique multicellular community. The biofilm formation process can generally be divided into four main phases (Fig 1): (i) initial attachment, (ii) division into microcolonies and production of extracellular matrix, (iii) maturation and cell proliferation, (iv) aging and dispersal (Zhou, & Cai, 2018; Magana, *et al.*, 2018). Biofilm formation is mainly regulated by QS, the signaling molecules used for QS can be very different (Schilcher & Horswill, 2020).

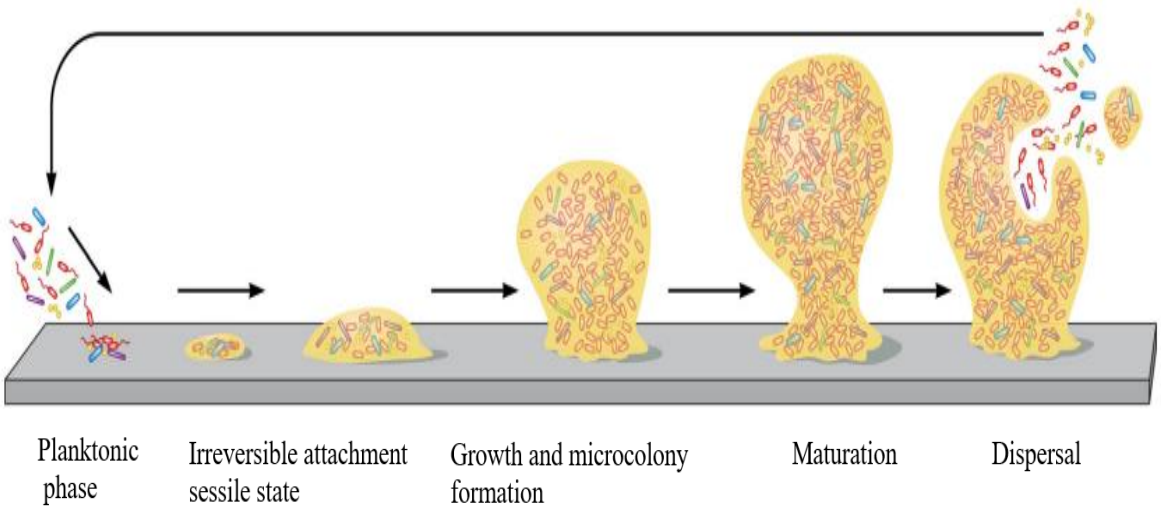


Figure 12: Biofilm formation developmental stages. Planktonic bacterial species attach to biotic/abiotic. Adherent bacteria grow as multicellular communities and form microcolonies that proliferate and mature. This microbial infrastructure leads to the development of mature Biofilms. Finally, biofilms act as bacteria Reservoirs that are brought back into the environment by biofilm diffusion and colonize new surfaces adapted from (Magana, *et al.*, 2018).

2.6.2. Inhibition of biofilm formation.

Targeting the attachment of bacterial cells and the development of biofilm structure is an ideal strategy (way) for inhibition of biofilm formation. QS Plays a key role in the formation of biofilm Natural Anti-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 in the quorum-sensing pathways (Mishra, *et al.*, 2020).

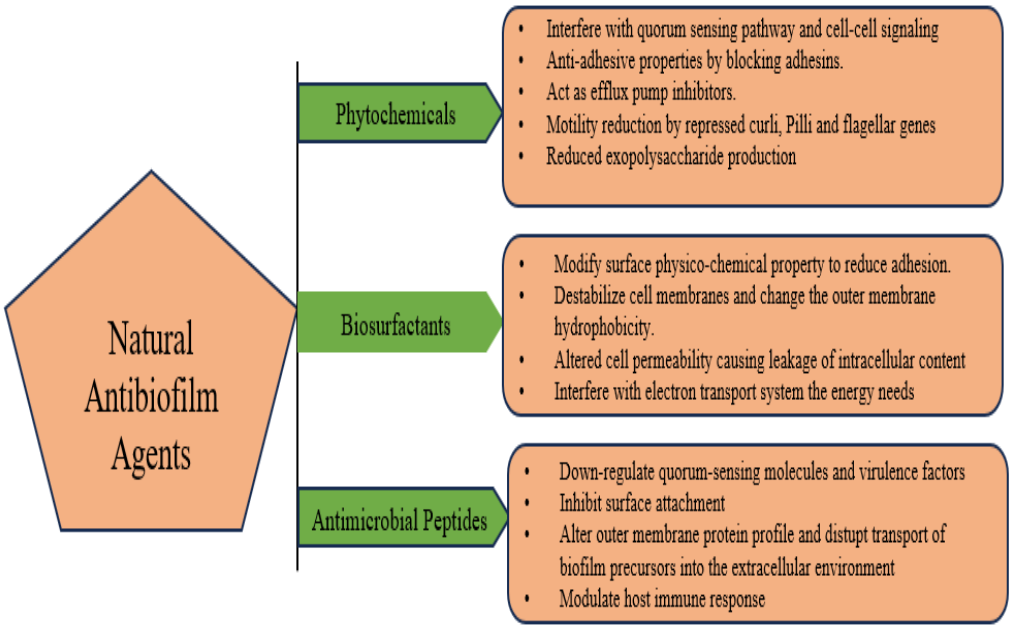


Figure 13: Flowchart review of natural antibiofilm agents adapted from (Mishra, *et al.*, 2020).

Natural compounds obtained from plants with potent antibiofilm properties are broadly classified into phenols, essential oils, terpenoids, lectins, alkaloids, polypeptides, and polyacetylenes. Phenols are a group of compounds consisting of seven subclasses including coumarins, flavones, flavonoids, flavonols, quinones, phenolic acids, and tannins (Yong, *et al.*, 2019). Flavonoids quercetin 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), which significantly inhibited 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. 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-infective that do not function by traditional bactericidal or bacteriostatic mechanisms (Paczkowski, *et al.*, 2017).

2.7. Inhibition of QS

Quorum sensing (QS) is the regulation of gene expression regulated by chemical signal molecules called auto-inducers (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; the signaling molecules used for QS can be very different. Two of the most studied systems are the Acyl-Homoserine Lactones' (AHL) QS systems in Gram-negative and Gram-positive (cyclic) peptide QS systems (Schilcher & Horswill, 2020). When the concentration 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, *et al.*, 2016).

CHAPTER THREE

3. MATERIALS AND METHODS

3.1. Description of sampling area

The plant leaves of *A. polystachyus* Delile were collected in early February (2023) from the Dilla University Botanical and Ecotourism Garden, which is located in the Southern Nations, Nationalities, and People's Region (SNNPR), Gedio Zone, Ethiopia. Dilla has a longitude and latitude of 6°24′30″N and 38°18′30″E, with an elevation of 1570 m above sea level, respectively. The plant material was authenticated by voucher specimen (Voucher No: HG001) and was deposited at the National Herbarium, Department of Botany, Addis Ababa University, Addis Ababa, Ethiopia.

Figure 14: *A. polystachyus* Delile Plant in Dilla University Botanical and Ecotourism Garden.

3.2. Apparatus (instruments)

The necessary materials and apparatus which were used in this study are Volumetric flasks, Beakers, Forceps, Autoclave, conical flasks, ruler, scissors, pipettes (different sizes), separatory funnel, water bath, electronic balance, test tubes, capillary tubes, funnels, digital measuring balance, measuring cylinders, Erlenmeyer flask, vials, plate reader, microtiter plate 96- well and nuclear were used for experiments.

3.3. Phytochemical extraction of *A. polystachyus* leaves

The leaves of *A. polystachyus* were collected from the field and washed with distilled water to remove dirt and air dried at room temperature (25-27°C) for two weeks. The dried leaves were coarsely grounded into powder by the analytical mill. The powdered plant material was stored in a plastic bag by wrapping it with aluminum foil until used for extraction. The leaf powder of *A. polystachyus* (184g) was transferred into a round bottom flask. Then, the coarse powder of the plant material was macerated with methanol 1:5 (v/v) for 72 h with occasional shaking, then filtered via Whatman filter paper No. 1. The residue was re-macerated for the second time with fresh same solvent, for a total of 6 d to obtain a better yield. The filtrate of the plant material obtained from the maceration was mixed and concentrated in a rotary evaporator (RE-501, Henan Lanphan Technology Co. Ltd., Henan, China), at 40°C. Finally, the crude extract of *A. polystachyus* leaves was kept separately and dried in a desiccate or until used for the next experiments. Then dried crude extract was stored in a refrigerator at -4^{o}C. The percentage extraction yield was calculated using the following formula (Anza, *et al.*, 2015):

$$\%\ \text{Crude extraction} = \frac{\text{weight of extract(w1)}}{\text{weight of plant material (w2)}} \times 100 \qquad \text{(Eq. 1)}$$

W1 is the crud extract obtained, and W2 is the weight of the plant sample before extraction.

3.4. Phytochemical analysis of *A. polystachyus* crude extract

3.4.1. Qualitative analysis of phytochemical components of *A. polystachyus* extract

The phytochemical screening tests were performed for se**c**ondary metabolite according to standard qualitative analysis:

3.4.1.1. Test for Alkaloids

Wagner's reagent: is prepared by dissolving 2 gm of iodine and 6 gm of potassium iodide in 100 mL of distilled water. The 1 mL of crude extract filtrate was treated with 2-3 drops of Wagner reagent (Otto Chemie Pvt. Ltd., Mumbai, India), and the formation of yellow color indicates the presence of alkaloids (Sbhatu & Abraha, 2020).

3.4.1.2. Test for Flavonoids

Ammonia Solution test: The crude extract (2 mL) was mixed with a few fragments of magnesium ribbon, followed by the dropwise addition of 5% concentrated HCl (CARLO ERBA Reagents S.A.S, Milano, Italy). The pink scarlet color indicates the presence and absence of flavonoids (Musyimi , *et al.*, 2021).

3.4.1.3. Test for glycosides:

To 2 ml of extract, 2 drops of Molisch's reagent (3.75g of alpha naphthol reagent is dissolved in a 25 ml solution of 99% ethanol) were added and shaken well. Then 2 ml of concentrated H_2SO_4 was added by the sides of the test tube. The formation reddish violet ring at the junction of two layers immediately indicated the presence of carbohydrates (Agbafor & Nwachukwu, 2011).

3.4.1.4. Test for Phenolics

Ferric chloride test: The 1 mL of crude extract filtrate was taken in a test tube, and 1-2 drops of iron III chloride ($FeCl_3$) (Loba Chemie Pvt. Ltd., Mumbai, India) were added and the color change (Agbaror & Namukobe, 2011).

3.4.1.5. Test for Saponins

Froth test: The filtrate of the crude extract (1 mL) was diluted with 5 mL of distilled water in a test tube. It was shaken by hand for 15 min. A foam layer on top of the test tube indicates the presence of saponins (Musyimi, *et al.*, 2021).

3.4.1.6. Test for Steroids

Salkowski's test: is a mixture of 15 ml 0.5 M FeCl3, 500 ml distilled water, and 300 ml of concentrated H_2S0_4 (CARLO ERBA Reagents S.A.S, Milano, Italy) sp. gr. 1.84. The crude extract filtrate (1 mL) was dissolved in 1 mL of chloroform in a test tube. Then, 1 mL of acetic anhydride and two drops of concentrated sulfuric acid were added to the test tube by the sides. The upper layer in the test tube turned red, indicating the presence of steroids (Oloya, *et al.*, 2022).

3.4.1.7. Test for Tannins

The plant extract filtrate (2 mL) was taken, and then a few drops of 10% (w/v) ferric chloride solution were added to the filtrate. The blue-green appearance indicates the presence of tannins in the sample (Agbaror & Namukobe, 2011).

3.4.1.8. Test for Terpenoids

Salkowski test: The crude extract filtrate (1 mL) was placed in a test tube and dissolved in chloroform (1 mL). The concentrated sulfuric acid (2 drops) was placed in the test tube and shaken. The lower yellow color indicates the presence of terpenoids (Agidew, 2022).

3.5. Screening of EO in *A. polystachyus* Delile

The EO from the leaves of *A. polystachyus* was extracted by hydro distillation in a Clevenger apparatus. A 316 g of powder from leaves was added in 1000 ml of distilled water using hydro distillation for 3 h in a modified Clevenger apparatus. The condensate (mixture of EO and water) was collected in a 100 mL separatory funnel. The water layer at the bottom was drained to separate the oil. The oil layer was treated with anhydrous sodium sulfate (Na_2SO4) to remove the remaining trace water. The EO was collected, consecutively separated from the aqueous layer, and dried using anhydrous sodium sulphate (Na_2SO_4). The obtained EO was protected from light and transferred into a GC-MS vial stored at -4°C until further analysis (Goudjil, *et al.*, 2020; Lemes, *et al.*, 2018). The phytochemical screening of *A. polystachyus* EO was done by UV spectrophotometry. A 100 µL of oil was diluted with 3 ml chloroform and UV spectra recorded the range of 200 nm- 400 nm to determine natural compounds (Añón, *et al.*, 2014; Anouar, *et al.*, 2012; Urbano, *et al.*, 2006).

Table 4: Screening of *A. polystachyus* EO natural compounds by using GC-MS.

Compounds	Range (nm)
Alkaloids	330-340
Coumarins	310-320
Flavonoids	290-300
Phenols	270-280

Terpenes	210-220

3.6. Gas Chromatography-Mass Spectra (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 (30m × 0.25 mm internal diameter (i.d.) and 0.25 μm film thickness). Helium was used as a carrier gas with a 4 min solvent delay and split less injection/purge time of 1.0 min with different flow rates and runtime. In the case of methanol extract, the temperature rise was 110-330 °C, the flow rate was 1.2 mL/min, and the run time was 60 min, respectively. For EO, the temperature rise was 100-280 °C, flow rate 1 mL/min, and run time was 33 min, respectively. Mass spectra were recorded in an electron-impact mode, with ionization energy of mode at 70 eV, scanning the 33-550 m/z range (Gülçin, *et al.*, 2004). The volatile compounds in the oil were identified by comparing the mass spectra of the compounds in oils with those in the database of NIST11 GC-MS libraries (Hanuš, *et al.*, 2008).

3.7. Anti-oxidant Activities

The antioxidant activity test was performed by using the DPPH free radical scavenging assay (Bondet, *et al*., 1997). A 0.1 mM DPPH solution was prepared in methanol and kept in the dark for 30 min to complete the reaction. The MeOH dilutions were prepared at 1000, 500, 250, and 125 µg/mL. The same concentrations of ascorbic acid were used as a standard (positive control) and the sample-free DPPH solution was used as a negative control, respectively. After mixing 1 ml of DPPH solution with 3 mL of prepared samples, the mixture was incubated at room temperature in a dark place for 30 min and the absorbance was measured at 517 nm. The assay for EO was similarly performed in Microtiter plates, except that the volumes were reduced. The %RSA was calculated according to the following formula.

$$\% \text{ RSA} = \frac{(\text{A0}) - (\text{A1})}{(\text{A0})} \times \ 100 \qquad \text{(Eq. 2)}$$

Where %RSA is the percent radical scavenging activity, A0 is the absorbance of the blank DPPH, and A1 is the absorbance of the sample (Bondet, *et al.*, 1997; Mengstu, *et al.*, 2023).

3.8. Evaluation of Antimicrobial Activity of the methanol extract and EO of *A. polystachyus* Delile.

The Antimicrobial activity tests were performed against standard organism strains **(**bacteria and fungus) obtained from the Adama Referral and Regional Research Laboratory. The test MOs included six pathogenic bacteria (three Gram-positive and three Gram-negative) and one fungus strains, respectively: *Streptococcus pyogenes* (ATCC 19615), *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginos*a (ATCC 27853), *Acinetobacter baumannii* (ATCC 19606), and *Candida albicans* (ATCC 10231) by the agar disk diffusion method.

Muller-Hinton broth (chemical composition Beef infusion solids, 2.0 g; Starch, 1.5 g; Casein hydrolysate, 17.5 g; Final pH 7.4 ± 0.2 at 25°C in 1L Distilled water), Muller-Hinton Agar (chemical composition Beef infusion solids, 2.0 g; Starch, 1.5 g; Casein hydrolysate, 17.5; Final pH 7.3 ± 0.1 at 25°C in 1L Distilled water), Potato dextrose agar (chemical composition Potatoes infusion solids, 200.0 g; Dextrose, 20 g; Agar, 15 g; Final pH 5.6 ±0.2 at 25°C in 1L Distilled water), Potato dextrose broth (chemical composition Potatoes infusion solids, 200.0 g; Dextrose, 20 g; Final pH 5.1 ±0.2 at 25°C in 1L Distilled water), Luria-Bertani(LB) broth (chemical composition Tryptone, 10 g; Yeast Extract, 5 g; NaCl, 10 g; Final pH 7.0 ±0.1 at 120°C in 1L Distilled water), and ketoconazole were from HI-Media Laboratories Pvt. Ltd., Mumbai, India; LB media (Bio Basic Inc., Ontario, Canada), gentamycin (Merck, Darmstadt, Germany), incubator (Thermo Stable SIR- 250, Daihan Scientific Co. Ltd., Gangwon-do, South Korea);

3.8.1. Preparation of standard inoculums

To determine the amount of microbes present, we used a McFarland standard with a chemical solution of Barium chloride and sulphuric acid, which resulted in the formation of a fine precipitate, barium sulphate. McFarland was shaken well and aliquot in test tubes identical to those used to prepare inoculum suspension. Before each use, shaking well was important for barium sulphate to evenly distribute in the solution. The strains of microbial cells were adjusted at 1.5×10^8 colony forming units (CFU/ml) which means 0.5 McFarland standard was prescribed

for the antimicrobial susceptibility test. This standard was prepared by mixing 0.05 ml $BaCl_2$ (1.17% w/v $BaCl_2$. $2H_2O$), 9.95 ml %1 w/v H_2SO_4 in distilled water, to standardize the approximate number of microbes in liquid suspension by comparing the turbidity of test with standard (Hudzicki, 2016; Mukhtar*, et al.,* 2017).

3.8.2. Antimicrobial Activity

The antimicrobial activity of MeOH and EO was evaluated by using a Kirby-Bauer agar disc diffusion assay on seven strains at various concentrations. The MeOH dilutions were prepared at 400, 200, 100, and 50 μg/mL by dissolving in 10% DMSO (v/v). The EO was prepared in three dilutions of 40, 20, and 10 μL/ mL by dissolving in 10% DMSO. First, all of the stock-cultured bacteria were activated in Muller-Hinton (MH) Broth, and Fungus in potato dextrose (PD) Broth. After activation, the cultures were subjected to inoculum development by inoculating a loop full of cells from a single colony into MH/PD agar and incubated for 24 h at 37(for bacteria) and 48 h at 30 °C (for fungus). The active pure bacterial/fungal cultures and subcultures were maintained according to the 0.5 McFarland standards to obtain 1.5×10^8 colony-forming units (CFUs/ mL) for bacteria and 1×10^6 (CFU/mL) for fungus. This process was repeated every time before a new experiment. The fresh overnight cultures of each strain were swabbed uniformly over sterilized and cooled MH/PD agar medium Petri dishes. Then, 6 mm diameter sterile discs impregnated with different concentrations of MeOH and EO were placed onto these plates, soaked for 30 min, and incubated for 24 h at 37 °C (for bacteria) and 48 h at 30 °C (for fungus). Discs impregnated with gentamycin solution (10 μg/mL), ketoconazole (30 μg/mL), and 10% DMSO which were used as positive and negative controls, respectively. After incubation, the antimicrobial activity was determined by measuring the diameter of the zone of inhibition. The antibacterial/antifungal activity was normalized and calculated in terms of percentage relative inhibition (%RI) by the following formula:

$$\%RI = \text{diameter of sample/diameter of control} \times 100$$

3.9. Minimum inhibitory concentration (MIC).

MIC was determined as the lowest concentration of extract that inhibited the visible growth of colonies of the organism (Mashamba*, et al.,* 2022). Bacterial growth was assessed by a broth microdilution method. MeOH was diluted in 10% DMSO, and the diluted extracts (100 μL)

were introduced into 10 mL of Luria-Bertani (LB) broth (for Bacterial) and Potato dextrose (PD) broth (for fungus) to achieve the concentrations of 1 mg/mL, 500 μg/mL, 250 μg/ mL, and 125 μg/mL. Inoculum (100 μL) was added from the McFarland standard calibrated broth (5 × 106 CFU/mL for bacteria and 1×10^4 for fungus) to each test tube and grown for 24 h at 37 °C for bacteria and 48 h at 30 °C for fungus. The difference between optical density at 600 nm (OD_{600}) at the end (6 h) and at the beginning (0 h) of incubation time in 96-well microtiter plates. 100µl of the dilution extracts were introduced in each well to be tested and 100µL of an inoculum (10^6 CFU/mL) was added at the same time then the plates were incubated at 37°C for 24hr and the MIC were calculated. The colony-forming unit CFU/mL was obtained to determine the viability of bacterial cells. For negative control, the extract was replaced with water (Trentin, *et al.*, 2013).

For EO, the growth assay was performed using the Broth microdilution method in a 96-well Microtiter plate. A 20 µl/mL stock solution was prepared in LB/PD broth. The concentrations were varied from 0.02-2.5 µl/ml in a final volume of 200 µl. 10 µl of McFarland calibrated (5×10^6 CFU/mL for bacteria and 1×10^4 for *C. albicans*) inoculum was added to each well and grown for 24 h at 37 °C for bacteria and 48 h at 30 °C for fungus.

3.10. Minimum bactericidal concentration (MBC) and Minimum fungicidal concentration (MFC).

MBC and MFC were the minimum concentrations corresponding to the lowest concentration of substance capable of killing more than 99.9% of bacterial inoculum (i.e. less than 0.1% of survivors). The plate assay for the determination of MIC were performed as mentioned above. The determination of MBC and MFC was also as above. So, we adopted a plate assay, where 20 μL of the above mixture was spread on MH/PD agar plates and incubated overnight at 37 °C for bacterial strains and 48 h at 30 °C for fungus. The gradual disappearance of colonies with increasing concentrations was observed visually and recorded. MBCs and MFCs of the extract for different microorganisms were deduced from the lowest concentration on the total inhibition of bacteria/*C. albicans* on the respective media agar. The operation will be done three times (Agbor, *et al.*, 2021; Trentin, *et al.*, 2013).

3.11. Antibiofilm Assay: Microtiter plate (Crystal violet stain)

The antibiofilm assay was performed only for bacteria with minor modifications (Ghasemi, *et al.*, 2013). The varying concentrations from MIC to sub-MIC (50-400 µg/ml for methanol extract and 0.02-1.25 µg/ml for EO) were mixed with bacterial culture media at an initial turbidity of 0.05 (5×10^5 CFU/mL). The assays were performed in a 96-well Microtiter plate for EO and 10 ml test tubes for MeOH as mentioned above. The mixtures were incubated for 48 h at 37 °C. 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) (Sisco Research Laboratories Pvt. Ltd., Mumbai, India); for 10 min and washed with PBS to remove the excess stain. After air drying, CV bound to biofilm was solubilized with 33% glacial acetic acid (Ghasemi, *et al.*, 2013; Kachhadia, *et al*., 2022). Using similar procedures, a control without any treatment was set for comparison to quantify the adherent cells, and the CV solution's absorbance was measured by a UV spectrophotometer (Optizen 2120UV, Mecasys Co. Ltd., Daegeon, South Korea) at OD_{590} and a microplate reader (PLATE READER, 8 Channel ELISA Photometer, DAS Italy SRL, Rome, Italy). The percent biofilm formation was calculated by

$$\text{Biofilm formation (\%)} = \frac{OD_{590}\ sample}{OD_{590}\ Control} \times 100\% \qquad \text{(Eq. 3)}$$

Minimum Biofilm Inhibitory Concentration (MBIC) was determined by inhibition of a minimum of 50% biofilm formation (Kachhadia, *et al*., 2022; Ghasemi, *et al.*, 2013).

3.12. Anti-QS assays: Pyocyanin Inhibition Assay

Varying dilutions (sub-MIC) of plant extracts were added to the planktonic cultures (6 ml) of *P. aeruginosa* and incubated for 24 h at 37 °C. The cultures were centrifuged at 4000 rpm for 20 min, and the supernatant was treated with 6.0 mL of chloroform followed by vigorous shaking. The chloroform layer was separated and solubilized by adding 2 mL of 0.2 N HCl and mixed gently. Then the OD of each supernatant of the HCl layer (cloudy layer) was measured at 520 nm against the suitable blank using a UV-VIS spectrophotometer (Shimadzu Europe - UV-2600) (Kachhadia, *et al.*, 2022; Tapia-Rodriguez, *et al*., 2017). The inhibition percentage is calculated as follows:

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

Where the percentage of Pyocyanin inhibition was determined by OD_{520} control and OD_{520} are the absorbance of the sample treated with DMSO and the one treated with the bacterial crude extract culture, respectively (Kachhadia, *et al.,* 2022).

3.13. Data analysis

The experimental results were expressed as mean ± standard deviation (SD) of three replicates. Where applicable, the data were subjected to Statistical Package of Social Science (SPSS 22.0)/PC+ version was used to analyze the experiment results, and the results were presented using tables and graphs. The differences between means were determined by using one-way ANOVA and the least significant difference test. The differences between means were determined by using one-way ANOVA and the least significant difference test. The level of statistical significance is set at $p \leq 0.05$. Microsoft Excel 2016 statistical package was used for all analysis.

CHAPTER FOUR

4. RESULTS

4.1. Phytochemical analysis of *A. polystachyus* Delile extract

4.1.1. Qualitative analysis of phytochemical components of *A. polystachyus* Delile extract

A. polystachyus leaves extract revealed by methanol 99.9% and maceration technique to extract the phytochemicals from *A. polystachyus* leaves Figure **14** (g).

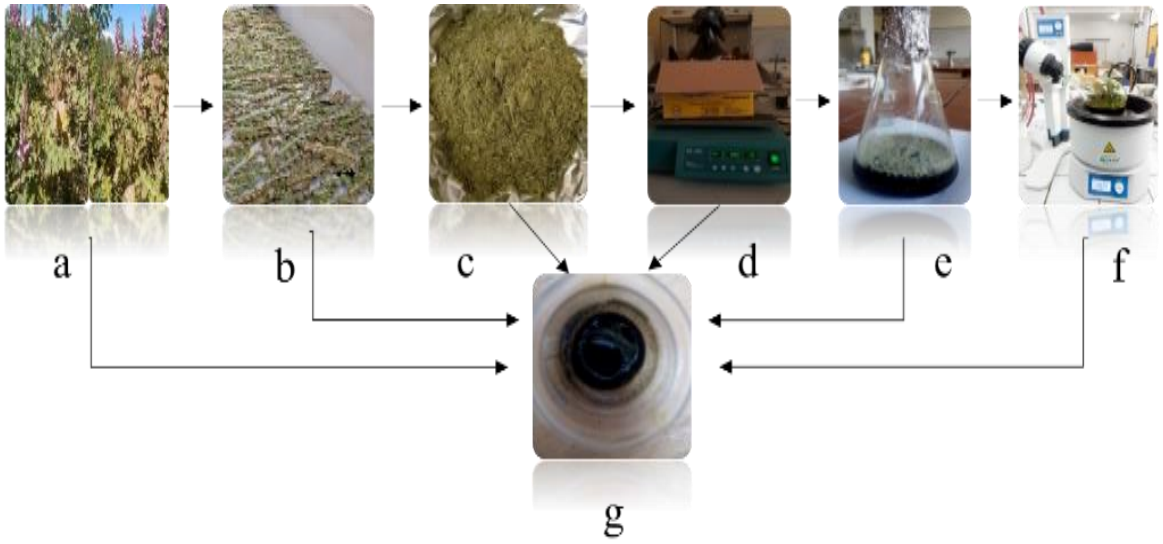


Figure 15: Phytochemical extraction process: a, *A. polystachyus* Delile plant in the Gedeo botanical garden b, dried leaves c, powder of leaves d, macerate the leaves in solvent e, the filtered extract f, the filtrate in Rotary evaporator to separate methanol from crud extract and g, the crud extract.

Phytochemical screening tests were conducted on the crude extract to identify the presence of different compounds like: Alkaloids, Phenols, Tannins, Saponins, Flavonoids, Steroids, and Terpenoids, except for Glycosides Table 5. This research provides valuable information about the phytochemical composition of *A. polystachyus* Delile extract, suggesting its potential for various biological activities and therapeutic applications.

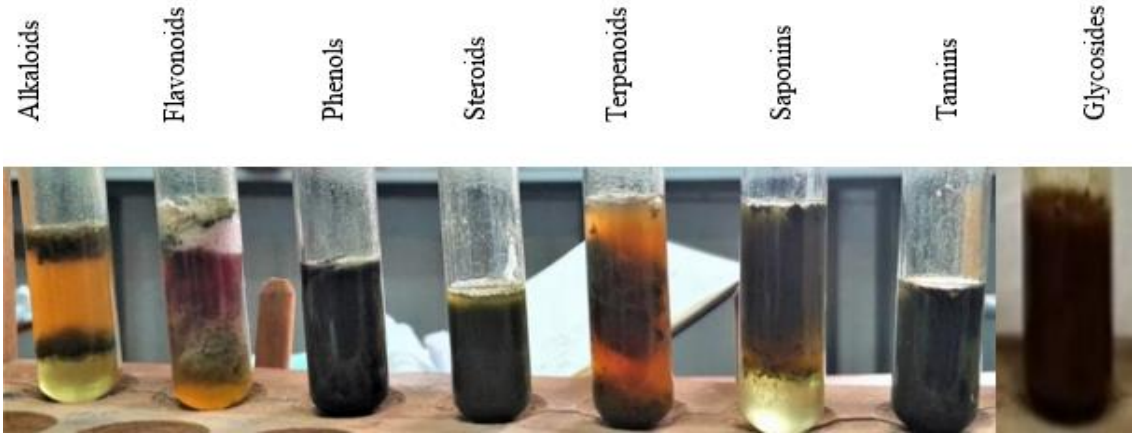


Figure 16: The phytochemical screening tests conducted on crude extract of *A. polystachyus* Delile leaves to the presence: of alkaloids, phenol, tannins, saponins, flavonoids, steroids, and terpenoids were present in the extract, Except for the extract glycosides.

Table 5: Preliminary phytochemical screening of crude leaves extracts of *A. polystachyus* Delile

Secondary Metabolites	Present of Metabolites
Alkaloid	+
Flavonoids	+
Glycosides	-
Phenols	+
Saponins	+
Steroids	+
Tannins	+
Terpenoids	+

(+): Indicates the presence of the constituents; (-): Indicates the absence of the constituents.

4.2. GC-MS Analyses of the Leave of *A. polystachyus* Delile

In the case of MeOH, GC-MS analysis was performed for 60 min that detected 79 compounds while the runtime for the *A. polystachyus* Delile of EO was light yellow and had a specific odor, it takes 33 min, and a total of 20 compounds were obtained. Some of the compounds appeared

twice or more as multiple peaks in MeOH and EO, while a stationary phase was observed after 29 min in the case of EO. The major compounds were identified based on the intensity of peaks that were generated in GC-MS chromatograms (Figure 17) and summarized in Table 6 (EO) and Table 7 (MeOH), respectively. The peak areas were used to calculate the individual compounds' percent occurrence (amount)**.**

The MeOH major compounds were found β-sitosterol acetate (16.06%), cholest-5-en-3-yl (9Z)-9-octadecenoate (9.54%), 1-dodecanol (7.57%), (S)-(E)-(-)-4-acetoxy-1-phenyl-2-dodecen-1-one (6.03%), Neophytadiene (5.7%), (E)-2-nonadecene (3.9%), hexanol-4-D2 (2.92%), and Decane (2.4%). The EO compounds detected by GC-MS were Stearyl alcohol P721 (25.38%); cis-9-tetradecenoic acid, isobutyl ester (22.95%); butyl 9-tetradecenoate (10.62%); 11, 13-dimethyl-12-tetradecen-1-ol acetate (10.14%); Ginsenol (3.48%); and Diisooctyl phthalate @P1404 (2.54%).

Table 6: GC-MS analysis of chemical composition against *A. polystachyus* Delile leaves for EO.

Formula	RT	Name	Percent abundance
$C_{18}H_{38}O$	25.201	Stearyl alcohol P721	25.37898205
$C_{18}H_{34}O_2$	28.863	cis-9-Tetradecenoic acid, isobutyl ester	22.95396431
$C_{18}H_{34}O_2$	28.511	Butyl 9-tetradecenoate	10.61806082
$C_{18}H_{34}O_2$	26.893	11,13-Dimethyl-12-tetradecen-1-ol acetate	10.13667091
$C_{18}H_{34}O_2$	25.981	Butyl 9-tetradecenoate	5.332813334
$C_{18}H_{38}O$	21.239	Stearyl alcohol P721	4.887969437
$C_{15}H_{24}O_2$	29.019	(2R,3R,4aR,5S,8aS)-2-Hydroxy-4a,5-dimethyl-3-(prop-1-en-2-yl) octahydronaphthalen-1(2H)-one	4.143525105
$C_{15}H_{26}O$	27.991	Ginsenol	3.483435105
$C_{24}H_{38}O_4$	23.671	Diisooctylphthalate @P1404	2.544659762
$C_{18}H_{38}O$	21.003	Stearyl alcohol P721	2.820067585
$C_{18}H_{34}O_2$	22.77	Butyl 9-tetradecenoate	2.161821191

$C_{24}H_{38}O_4$	18.606	Phthalic acid, butyl dodecyl ester	0.806374408
$C_{15}H_{24}O_2$	23.497	(2R,3R,4aR,5S,8aS)-2-Hydroxy-4a,5-dimethyl-3-(prop-1-en-2-yl) octahydronaphthalen-1(2H)-one	0.552747042
$C_{26}H_{42}O_4$	17.664	Phthalic acid, butyl tetradecyl ester	0.559619801
$C_{17}H_{32}O_2$	26.125	i-Propyl 9-tetradecenoate	0.488689087
$C_{15}H_{24}O_2$	25.709	(2R,3R,4aR,5S,8aS)-2-Hydroxy-4a,5-dimethyl-3-(prop-1-en-2-yl) octahydronaphthalen-1(2H)-one	0.303467407
$C_{14}H_{22}O$	13.627	Phenol, 2,4-bis (1,1-dimethyl ethyl)-	0.29127704
$C_{15}H_{24}O$	16.504	(3S,3aR,6R,8aS)-7,7-Dimethyl-8-methyleneoctahydro-1H-3a,6-methanoazulen-3-yl) methanol	0.28997272
$C_{18}H_{34}O_2$	25.432	cis-9-Tetradecenoic acid, isobutyl ester	0.289496142
$C_{10}H_{14}O$	10.734	Phenol, 5-methyl-2-(1-methyl ethyl)-	0.289362366
$C_8H_{16}O_2$	8.909	Octanoic acid	0.221525229
$C_{14}H_{28}$	18.819	Cyclo tetradecane P391	0.203891837
$C_{15}H_{24}O_2$	21.586	(2R,3R,4aR,5S,8aS)-2-Hydroxy-4a,5-dimethyl-3-(prop-1-en-2-yl) octahydronaphthalen-1(2H)-one	0.197830098
$C_{13}H_{22}O$	13.402	3-Buten-2-ol, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-	0.182943301
$C_{19}H_{38}$	14.557	(E)-2-nonadecene	0.181429957
$C_{11}H_{14}O_2$	13.252	Ethanone, 1-[4-(1-hydroxy-1-methyl ethyl) phenyl]-	0.162178708
$C_{18}H_{34}O_2$	24.722	11,13-Dimethyl-12-tetradecen-1-ol acetate	0.133751239
$C_{12}H_{24}O_2$	14.147	Lauric acid P400	0.129064888
$C_{15}H_{24}O_2$	24.098	(2R,3R,4aR,5S,8aS)-2-Hydroxy-4a,5-dimethyl-3-(prop-1-en-2-yl) octahydronaphthalen-1(2H)-one	0.127309073
$C_{12}H_{11}ClN_2O_2S$	6.235	Batzelline A	0.127100048

Table 7: GC-MS analysis *of* chemical composition for *A. polystachyus* Delile leaves MeOH extract.

Formula	RT	Name	Percent abundance
$C_{14}H_{30}$	24.528	Tetradecane P396	0.4413771
$C_{15}H_{30}$	24.699	4-Isopropyl-1,7-dimethyl cyclo decane	0.9348913
$C_{18}H_{30}O_3$	24.997	(2-pentyl cyclopentyl) 2-cyclohexyl-2-oxidanylidene-ethanoate	0.220978
$C_8H_{11}F_2P$	25.352	Dimethyl(phenyl) difluoro phosphorane	0.0375959
$C_{24}H_{20}N_2O_6$	25.603	[2,4-dimethyl-6-nitro-2-(3-nitrophenyl)-3,4-dihydro-2H-1-benzopyran-3-yl]-phenyl methanone	0.0315683
$C_8H_{13}N$	25.718	Cyclohexane acetonitrile	0.1166018
$C_{14}H_{28}$	25.97	Cyclohexane, octyl-	1.111071
$C_{12}H_{14}O_3S_2$	26.45	Ligand3-Methylmercapto-3'-oxomethylmercapto-1-(p-methoxyphenyl)-2-propen-1-one	0.0744596
$C_{18}H_{26}O_3$	26.713	(S)-3-(2,3-Dimethoxy-5-methyl phenyl)-4,4-dimethylhepta-1,6-diene-3-ol	0.0238891
$C_{13}H_{18}O$	27.177	4-(2,6,6-Trimethylcyclohexa-1,3-dienyl) but-3-en-2-one	1.69488
$C_8H_{11}NO$	27.858	2-Methyl-4-cyanocyclohexanone	1465354
$C_{20}H_{28}O_2S$	28.007	7,7-Dimethyl-4-(phenylsulfonyl)-3-methylene-1-methyl-bicyclo [4.4.0] octane	0.0848291
$C_{14}H_{28}$	28.779	Cyclohexane, 1,2,4,5-tetraethyl-, (1. alpha.,2.alpha.,4.alpha.,5.alpha.)-	0.1879284
$C_{14}H_{18}O_2$	28.859	(-)-(1S,2S,3R)-2,3-Epoxy-4-methyl-1-phenyl-3-vinyl-1-pentanol	0.2260351
$C_{21}H_{34}O_2S$	29.162	(E)-4-Tosyl-5-tetradecene	0.2138947
$C_{17}H_{34}O_2$	29.557	[(2R,3R)-3-myristyloxiran-2-yl] methanol	0.3778489
C15H30O	29.695	1-Pentadecen-4-ol	0.1235488

$C_{13}H_{18}O$	30.032	Megastigmatrienone	1.1976539
$C_{12}H_{26}O$	30.347	1-Dodecanol	7.5738185
$C_{18}H_{35}F_3O$	30.553	1,1,1-trifluoro-2-octadecanol	0.4456169
$C_{12}H_{14}FNO$	30.936	Cyclopentane carboxamide, N-(4-fluorophenyl)-	0.0335094
$C_{19}H_{38}$	31.028	(Z)-2-nonadecene	0.2754478
$C_{13}H_{18}O$	31.354	(4Z)-4-[(E)-but-2-enylidene]-3,5,5-trimethyl-1-cyclohex-2-enone	0.5794501
$C_{16}H_{32}$	32.098	Cyclohexane, decyl-	0.7092479
$C_{16}H_{14}N_4S_2$	32.464	2-p-toludinomethyl-3-thion-1,2,4-triazolo[3,4b] benzothiazole	0.0293037
$C_{15}H_{30}O$	32.619	8-Pentadecanone	1.5814113
$C_{20}H_{19}NO_5S$	33.82	3-(Benzyloxycarbonyl)-1-(p-toluenesulfonyl)-5,6-dihydro-2(1H)-2-pyridone	0.1734724
$C_{24}H_{36}O_9$	34.741	Desacylin-Caspitolide D -5-[O-(2'-Methylbutyrate)]	0.0824964
$C_{11}H_{17}N_3O$	35.354	2-(2-cyanoethyl)-3,3-diethyl-4-isoxazolidinecarbonitrile	0.016295
$C_{19}H_{38}$	35.823	(E)-2-nonadecene	3.9033152
$C_{23}H_{35}ClO_2Si_2$	36.876	3,6-Dioxa-2,7-disilaoctane, 4-(4-chlorophenyl)-2,2,4,5,7,7-hexamethyl-5-(4-methylphenyl)-	0.0187809
$C_{20}H_{38}$	37.013	Neophytadiene	3.2309481
$C_{12}H_{24}O$	37.631	Dodecanal	0.7625257
$C_{15}H_{30}O$	37.906	8-Pentadecanone	1.4606549
$C_{20}H_{40}O$	38.089	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	1.3982846
$C_{18}H_{34}$	38.936	Cyclohexane, 1,1'-(2-propyl-1,3-propanediyl) bis-	0.157552
$C_{17}H_{34}O$	39.079	(E)-2-heptadecen-1-ol	0.1477103
$C_{17}H_{34}O_2$	39.199	Hexadecanoic acid, methyl ester	0.389172
$C_{32}H_{66}O$	39.577	1-Dotriacontanol	0.0867532
$C_{13}H_{19}NO_4S$	40.109	(E)-2,6-dimethyl-7-(2-methyl-1,3-thiazol-4-yl)-3,5-bis (oxidanyl) hept-6-enoic acid	0.1797894

$C_{19}H_{38}$	40.795	(E)-2-nonadecene	1.0954571
$C_{18}H_{38}O_2$	42.438	1,12-Octadecanediol	0.1084117
$C_{32}H_{54}O_2$	42.707	1,2-bis[1,2,3-tri(t-Butyl)-2-cyclopropen-1-yl] 1,2-ethanedione	0.102929
$C_{32}H_{56}N_4$	43.284	1,4-Dihydro-3,6-bis[1,2,3-tri(t-butyl)-2-cyclopropen-1-yl] 1,2,4,5-tetrazine	0.0789718
$C_{20}H_{38}$	43.553	Neophytadiene	5.7074583
$C_{21}H_{26}O_6$	44.217	Phenyl 4-[bis(ethoxycarbonyl)but-3-ynyl]-2,3,4-trideoxy-.alpha.,L-glcero-pent-2-enopyranoside	0.2032187
$C_{16}H_{30}$	45.333	Cyclohexane, 1,1'-(2-methyl-1,3-propanediyl) bis-	0.2784786
$C_{18}H_{28}O_2$	48.343	(9Z,11E,13S,15Z)-Octadeca-9,11,15-trien-13-olide	1.5010092
$C_{45}H_{78}O_2$	49.15	Cholest-5-en-3-yl (9Z)-9-octadecenoate	4.4833272
$C_{20}H_{28}O_3$	51.473	(S)-(E)-(-)-4-Acetoxy-1-phenyl-2-dodecen-1-one	6.0307183
$C_{45}H_{78}O_2$	55.564	Cholest-5-en-3-yl (9Z)-9-octadecenoate	9.5475859
$C_{31}H_{52}O_2$	56.697	.beta.-Sitosterol acetate	16.063543
$C_{20}H_{28}O_3$	58.299	(S)-(E)-(-)-4-Acetoxy-1-phenyl-2-dodecen-1-one	5.5525966

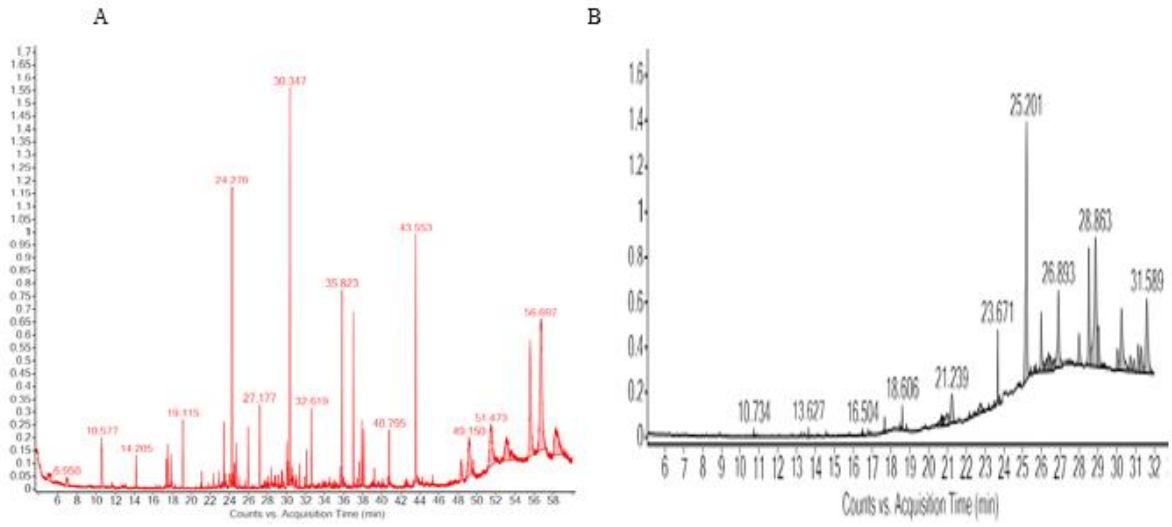


Figure 17: GC-MS Chromatographic profile: a. MeOH (red) and b. EO (black) of *A. polystachyus* Delile. The peaks indicate the major compounds and the numbers above the peaks depict the retention time. The y-axis is for "Intensity".

4.3. Antioxidant Scavenging Assay

The DPPH free radical scavenging abilities of the *A. polystachyus* Delile were studied significantly differently ($p < 0.05$) and results have been presented in Table 8. Ascorbic acid was used as a standard drug determination of the antioxidant activity by the DPPH method. The concentration of ascorbic acid and the *A. polystachyus* of MeOH and EO varied from 125 to 1000 µg/mL. The percentages scavenging of the extract of MeOH and EO showed an increasing order of concentration: "125" (48.3%) < "250" (65.7%) < "500" (76.5%) < "1000" (84.2%); "125" (45.6%) < "250" (61.3%) < "500" (71.0%) < "1000" (82.0%) respectively. Likewise, the percentages scavenging of ascorbic acid were: "125" (90.5%) < "200" (91.0%) < "500" (92.4%) < "1000" (94.7%) concentration of the standard drug.

Table 8: Antioxidant activity of MeOH and EO of *A. polystachyus* Delile.

concentration (µg/mL)	MeOH (%RSA)	EO (%RSA)	standard (%RSA)
125	48.3	45.6	90.5
250	65.7	61.3	91
500	76.5	71.6	92.4
1000	84.2	82	94.7

[a] Standard, ascorbic acid. For EO, the concentration is in µL/mL.

4.4. Antimicrobial activities of *A. polystachyus* Delile Leaves extract

The antimicrobial potential of MeOH and EO was evaluated by using a Kirby-Bauer agar disc diffusion assay on six bacterial and one fungal species at various concentrations (50-400 mg/mL) of MeOH and (10-40 mg/mL) of EO. The microorganisms were chosen based on their pathogenic relevance and AMR potential, including three Gram-negative bacteria, *P. aeruginosa*

(ATCC 27853), *E. coli* (ATCC 25922), and *A. baumannii* (ATCC 19606); three Gram-positive bacteria, *S. aureus* (ATCC 25923), *E. faecalis* (ATCC 29212), and *S. pyogenes* (ATCC 12204); and one fungal strain, *C. albicans* (ATCC 10231). At the tested concentrations, both MeOH and EO inhibited all of the microbes with varying degrees of inhibition (Figure 18 and Tables 9 and 10). The antimicrobial assay of MeOH shown in Figure 18 indicates that both Gram-positive and Gram-negative bacteria were inhibited with dose dependency.

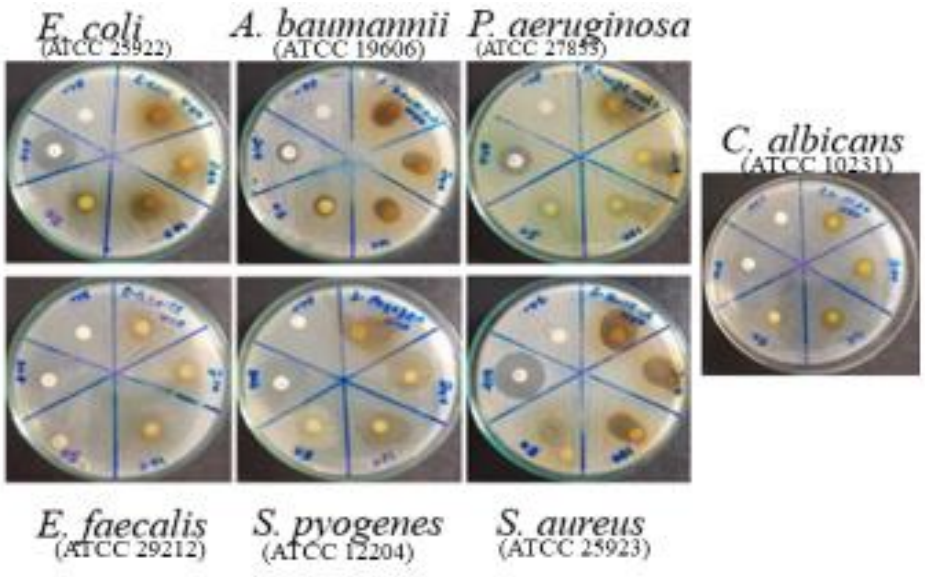


Figure 18: Antimicrobial activity of methanol extract by the disk diffusion assay. Bacteria and fungi were treated with varying concentrations of MeOH (50−400 mg/ mL) on discs. Gentamycin (10 μg/mL) was the positive control for bacteria and ketoconazole (30 μg/mL) for *C. albicans* (ATCC 10231). The negative control was 10% DMSO.

Similarly, inhibition increased with increasing concentration in the case of *C. albicans*. The zone of inhibition (ZOI) was measured and is presented in Tables 9 (MeOH) and 10 (EO). Due to the differential behavior of microorganisms toward antibiotics, inhibition zones were normalized with control (%RI, relative inhibition). There was no apparent difference in the inhibition patterns between the Gram-positive and Gram-positive bacteria, indicating the broad-spectrum inhibitory activity of the extracts. In Gram-positive representative bacteria, inhibition was observed in the 32.72-87.75% range, while in Gram-negative representative strains, a wide range of inhibition (39.2-130.2%) was observed (Table 9).

Table 9: Antimicrobial activity of Disc Diffusion Assay (mm) of *A. polystachyus* Delile [a] of MeOH crude extract.

Bacteria	Mean inhibition zone(mm) ± SD and % inhibition Concentration (mg/ml) +ve control	50(% RI)	100 (% RI)	200(% RL)	400 (% RI)
ATCC 27853	12.6 ± 0.33	8.0 ± 1.00 (63.5%)	8.5 ± 1.33 (67.46%)	9.3 ± 0.50 (74.05%)	12.8 ± 0.33 (100.6%)
ATCC 25922	17.0 ± 0.57	6.6 ± 0.33 (39.2%)	7.3 ± 0.33 (43.12%)	7.3 ± 0.33 (43.12%)	8.3 ± 0.33 (49%)
ATCC 19606	9.4 ± 0.56	7.3 ± 0.33 (77.4%)	10.3 ± 0.66(106.3%)	10.7 ± 0.57 (111.9%)	12.3 ± 0.33 (130.2%)
ATCC 12204	19.3 ± 0.66	6.6 ± 0.33 (32.7%)	9.0 ± 0.57 (43.1%)	13.3 ± 0.88 (60.40%)	18.6 ± 1.2(87.75%)
ATCC 29212	21.6 ± 1.20	8.3 ± 0.88 (38.4%)	10.6 ± 0.88 (49.2%)	13.6 ± 2.40 (63.08%)	14.6 ± 2.33(67.7%)
ATCC 10231	19.0 ± 0.58	6.0 ± 0.33 (40.3%)	8.6 ± 0.66 (45.6%)	10.0 ± 1.15 (52.63%)	12.0 ± 1.15 (63.16%)
	25.3 ± 0.33	8.7 ± 0.33 (34.2%)	10.3 ± 0.33 (40.8%)	12.3 ± 0.33 (48.68%)	15.0 ± 0.57 (59%)

[a] % RI (% relative inhibition); + ve control, positive control. The relative percentage zone of inhibition (% RI) of the compounds was calculated as (ZOIc - ZOIe)/ (ZOIc) × 100%, where ZOIc is the mean inhibition zone in the Petri dish plate disc of positive control under investigation, ZOIe is the mean inhibition zone in the Petri dish plate disc with extract (compounds).

Antimicrobial activity of the crud extract based on concentration difference relative percentage zone of inhibition (% RI) of the compounds was calculated as (ZOIc - ZOIe)/ (ZOIc) × 100%, where ZOIc is the mean inhibition zone in the Petri dish plate disc of positive control under investigation, ZOIe is the mean inhibition zone in the Petri dish plate disc with extract (compounds). The antimicrobial activity of EO at various concentrations (10-40 μL/mL) is presented in Table 10. The EO inhibited Gram-positive (34-74%) and Gram-negative (40.2-89.8%) representative bacteria generally without any apparent difference.

Table 10: Antimicrobial activity of EO by Disc Diffusion Assay

	mean inhibition zone (mm) ± SD and % inhibition concentration(µL/mL)			
Bacteria	**+ve control**	**10 (%RI)**	**20 (%RI)**	**40 (%RI)**
ATCC 27853	13.2 ± 0.50	6.9 ± 1.2 (52.2%)	7.3 ± 1.1 (55.3%)	9.6 ± 0.20 (72.7%)
ATCC 25922	16.4 ± 0.57	6.6 ± 0.66 (40.2%)	7.5 ± 0.33 (45.7%)	8.2 ± 0.50 (50.0%)
ATCC 19606	9.9 ± 0.85	6.8 ± 0.33 (68.7%)	8.1 ± 0.26 (81.8%)	8.9 ± 0.33 (89.8%)
ATCC 25923	18.5 ± 0.33	6.3 ± 0.4 (34.0%)	9.2 ± 0.60 (49.7%)	13.7± 0.38 (74.0%)
ATCC 12204	22.0 ± 1.35	8.5 ± 0.7 (38.6%)	10.0 ± 0.33 (45.4%)	13.9 ± 2.6 (63.18%)
ATCC 29212	19.6 ± 0.75	7.6 ± 0.2 (38.7%)	8.7 ± 0.33 (44.38%)	11.3 ± 1.0 (57.65%)
ATCC 10231	22.7 ± 0.46	7.5 ± 0.50(33.0%)	9.4 ± 0.85 (41.4%)	12.2 ± 0.20 (53.7%)

[a] % RI, % relative inhibition; + ve control, positive control.

4.5. Minimum inhibitory concentration (MIC), Minimum bactericidal concentration (MBC), and minimum fungicidal concentration (MFC) of MeOH and EO.

Determining the MIC, MBC, and MFC is necessary for evaluating the extracts for their cytotoxicity. The MIC/MBC/MFC assays were carried out for MeOH and EO; the results are presented in Table 11. A tolerance test provides information about the potency of the test extracts, where lower values indicate bactericidal potential and higher values indicate bacteriostatic activity.

4.5.1. Minimum inhibitory concentration (MIC)

In the present study, the MIC value obtained from *A. polystachyus* Delile extract and EO showed a difference in the inhibitory concentrations against test microbes. Thus, the material's effectiveness at low concentrations is important to determine its potential as antimicrobials. The MIC values of MeOH against the tested microbes varied from 0.5 to 1.0 mg/mL. The MIC values of EO for the tested microbes varied from 0.31 to 0.62 μL/mL. MICs were determined using the microdilution method. The 96 well Microtiter plates were used and each plant extract was tested in triplicates at serial dilutions for crud extract and EO of *A. polystachyus* Delile,

which were found better than the MIC values of the extract. MICs for fungi and bacteria were determined by visual inspection. *E. coli* recorded an MIC of 1 mg/mL, which was higher than other bacteria, indicating higher resistance. Similar resistance patterns were observed in the antibacterial assay for *E. coli* (Figure 18 and Table 11). There was no other apparent difference observed among the strains, and it indicated the broad-spectrum activity of the extracts. *P. aeruginosa* and *C. albicans* showed higher values MIC of 0.62 μL/mL.

4.5.2. Minimum bactericidal concentration (MBC) and Minimum Fungicidal Concentration (MFC).

MBC/MFC is the lowest concentration required to kill the bacterium/fungus. Thus, the material's effectiveness at low concentrations is important to determine its potential as antimicrobials. A tolerance test provides information about the potency of the test extracts, where lower values indicate bactericidal potential and higher values indicate bacteriostatic activity. The MBCs/MFCs values of MeOH against the tested microbes varied from 2 to 4 mg/mL.

Table 11: MIC, MBC, MFC, MBC/MIC, MFC/MIC, and MBIC of MeOH and EO of *A. polystachyus* Delile [a].

Bacteria		MIC (mg/mL)	MBC (mg/mL)	MFC (mg/mL)	MBC/MIC	MFC/MIC	MBIC (mg/mL)
ME	ATCC 27853	0.5	2		4		0.5
	ATCC 25922	1	4		4		1
	ATCC 19606	0.5	4		8		0.5
	ATCC 25923	2	4		0.5		
	ATCC 19615	0.5	2		4		0.5
	ATCC 29212	0.5	4		8		0.5
	ATCC 10231	0.5		4		8	
EO	ATCC 27853	0.62	2.5		4		0.62
	ATCC 25922		1.25		4		0.31
	ATCC 19606	0.31	1.25		4		0.31
	ATCC 25923		1.25		4		0.31
	ATCC 19615	0.31	1.25		4		0.31
	ATCC 29212	0.31	1.25		4		0.31
	ATCC 10231	0.62		2.5		4	

[a]MeOH, methanol extract; EO, values are in μL/mL; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; MFC, minimum fungicidal concentration; MBC/MIC, tolerance values for bacteria; MFC/MIC, tolerance values for fungi; MBIC, minimum biofilm inhibitory concentration (MBIC). The values are from three independent experiments ($P < 0.05$).

A. B.

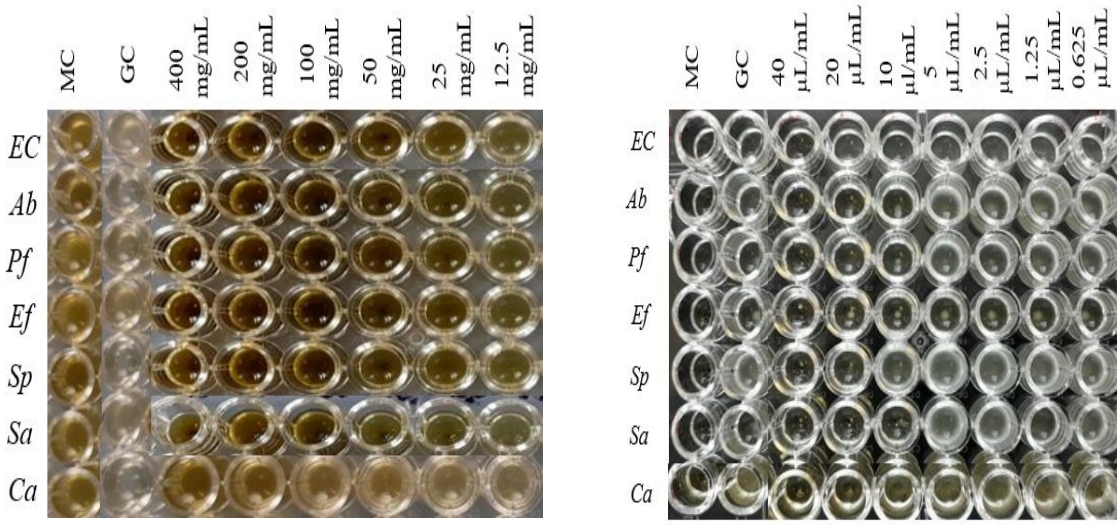


Figure 19: MIC, MBC/ MFC on Microtiter plate: a, MeOH of crude extraction; b, EO, *Ec: E coli, Pa: P. aeruginosa, Ab: A. baumannii, Sp: S. pyogenes, Sa: S. aureus, Ef: E. faecalis* and *Ca: C. albicans*.

Tolerance test values calculated as MBC/MIC (for bacteria) and MFC/MIC (for fungi) were between 4 and 8. When the value of < 4 it indicates bactericidal activity, while a value of 8 indicates bacteriostatic action. The strains *A. baumannii*, *E. faecalis*, and *C. albicans* recorded a value of 8, indicating bacteriostatic/fungistatic activity, while all others scored 4 indicating the bactericidal potential of MeOH and EO. MBCs/MFCs from 1.25 to 2.5 μL/mL. *P. aeruginosa* and *C. albicans* showed higher values. MBC/MFC of 2.5 μL/mL, indicating higher resistance. All strains scored tolerance test values of 4, indicating EO bactericidal/fungicidal activity.

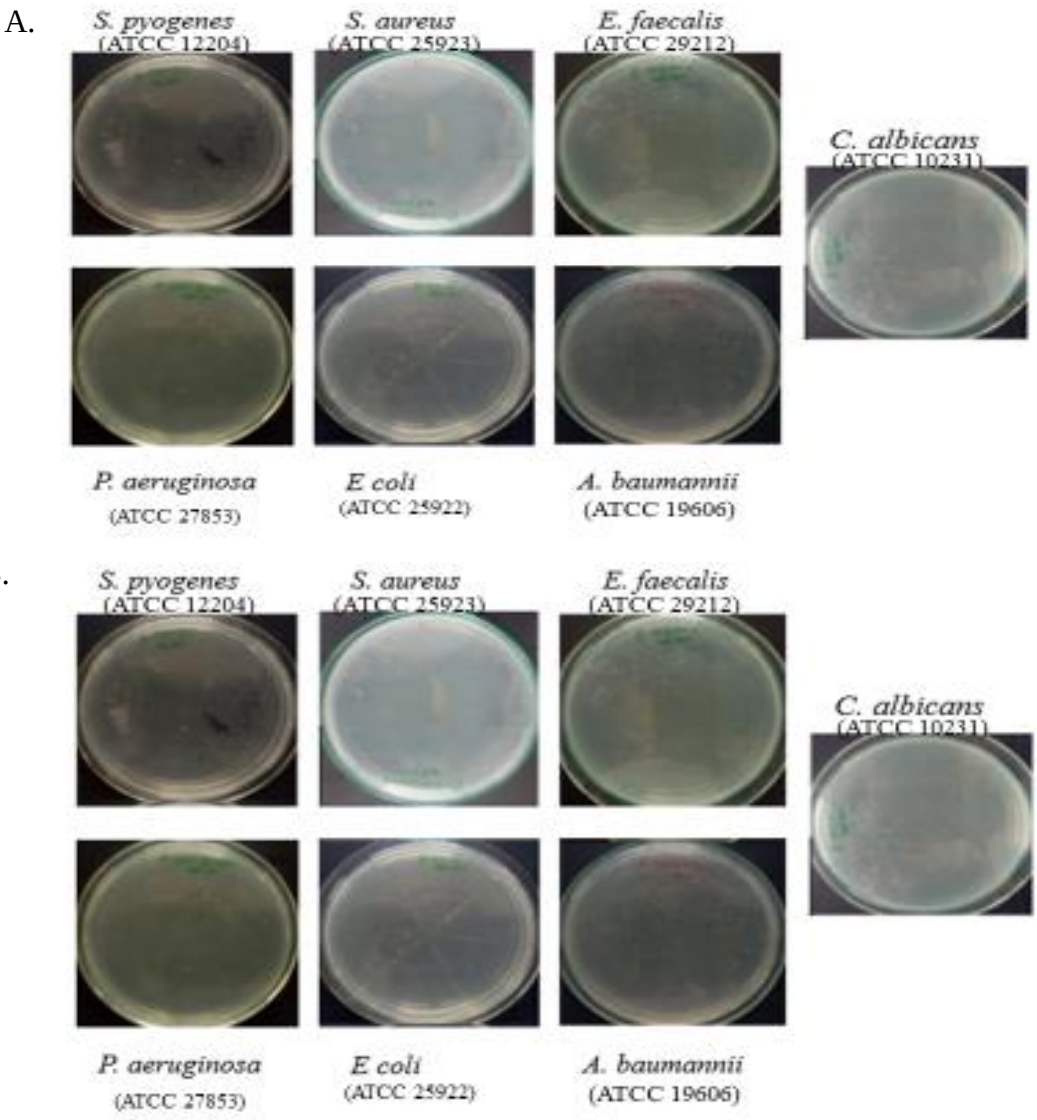


Figure 20: The MBC of A, MeOH, and B, of EO, by the spread of the bacteria and fungi were treated with varying concentrations on discs.

4.6. Antibiofilm assay and minimum biofilm inhibition concentration (MBIC) of MeOH and EO (Crystal violet stain).

Microbes develop resistance when challenged by conventional anti-microbials, leading to the impaired treatment of diseases. The resistance is most commonly associated with forming biofilms that protect microbes from surrounding environmental stresses, impede phagocytosis, and confer the capacity for colonization and long-term persistence. Such an ability is promoted by effective cell-to-cell communications (quorum sensing) within the microbial communities.

As a result, highly structured biofilms can be formed and often identified in patients with chronic infections, such as chronic lung and wound infections. Therefore, exploring new sources with pharmaceutical properties that can interfere with, reduce, and eradicate biofilms for the effective treatment of such diseases is essential. The MeOH and EO of *A. polystachyus* were investigated for their ability to reduce biofilm formation. The results are shown in Figure 21 and Table 11. Both ME and EO were effective and showed dose-dependent inhibition of biofilm formation, with MBIC values in the range of 0.5-1 mg/mL (MeOH) and 0.31-0.62 µL/mL, respectively. The Gram-negative representative bacteria were more resistant to the biofilm inhibitory effect of MeOH and EO than Gram-positive strains. At the highest concentration, 1 mg/mL of MeOH, the biofilm inhibitory effect was in the following order: *E. faecalis* > *S. pyogenes* > *S. aureus* > *A. baumannii* > *E. coli* > *P. aeruginosa*. In the case of EO, the following order was observed: *S. pyogenes* > *E. faecalis* > *S. aureus* > *P. aeruginosa* > *E. coli* > *A. baumannii*. The reduction of the biofilm formation ability of microbes has been reported to vary across an extensive range (0.125-100 mg/mL).

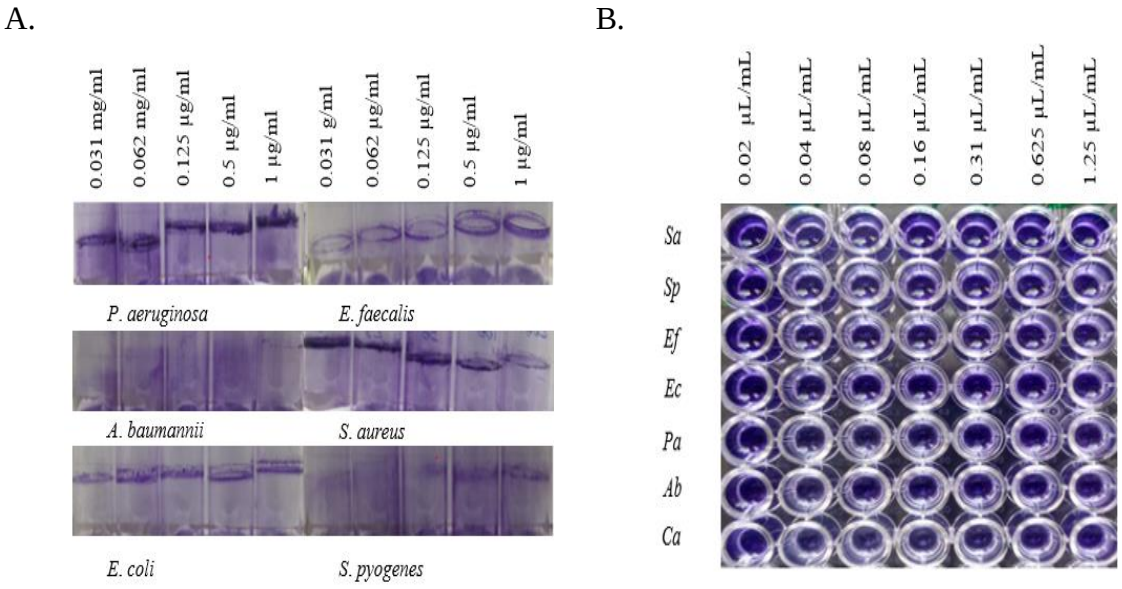


Figure 21: Antibiofilm of A. Plant extract (Crystal violet stain) on test tube B. Microtiter plate biofilm assay of Essential oil (Crystal violet stain) EO, *Sa: S. aureus, Sp: S. pyogenes, Ef: E. faecalis, Ec: E. coli, Pa: P. aeruginosa,* and *Ab: A. baumannii*.

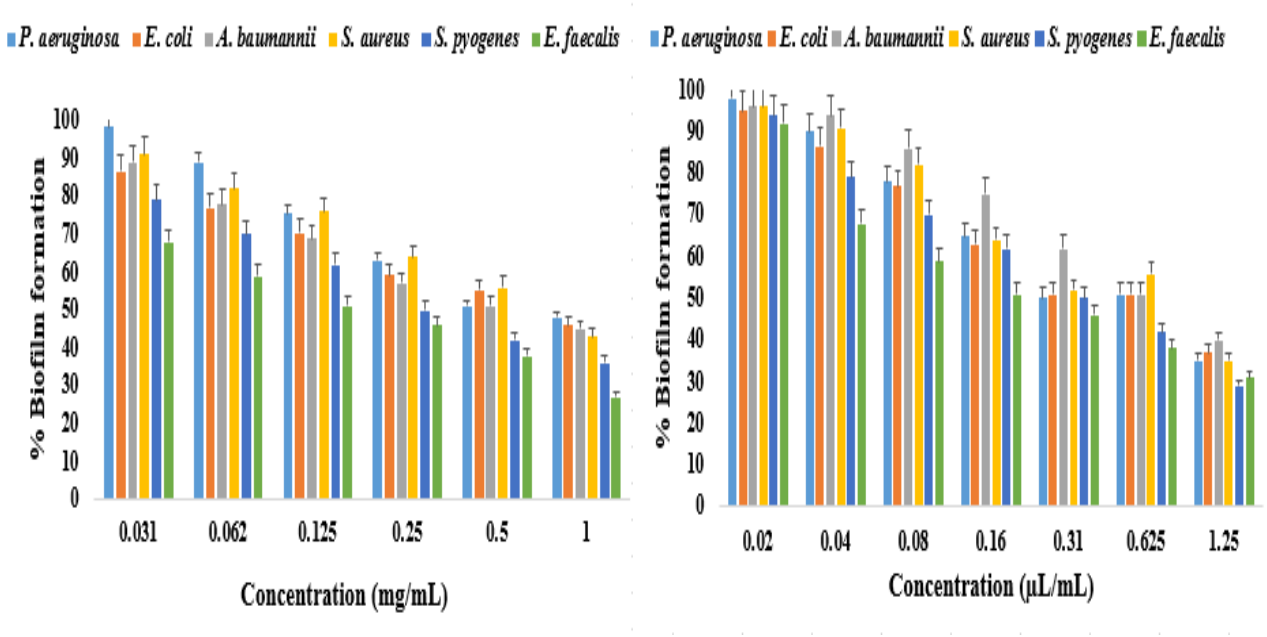


Figure 22: Antibiofilm assay of MeOH and EO. The biofilm formation ability of bacteria was tested using different concentrations of MeOH (left) and EO (right). The %biofilm formation was inhibited by increasing the extract concentrations in a dose-dependent manner. Values are from three independent experiments ($P<0.05$).

4.7. Antiquorum sensing Activity of MeOH

4.7.1. Pyocyanin assay

The Pyocyanin inhibition ability of the crude extract was tested using different bacterial species. Planktonic of 10 ml LB broth treated with different dilutions of plant extract and incubated with little modifications, at 37 °C for 24 h. Pyocyanin inhibition assay (PIA) is commonly employed to evaluate quorum sensing (QS) in the model bacteria, *P. aeruginosa* (Pejin, *et al.*, 2020). In this study, we evaluated the anti-QS potential of MeOH on *P. aeruginosa* by using PIA. The inhibition of pyocyanin production was in the range of 17.4-51.6%, indicating the effective inhibition of pyocyanin (Figure 23).

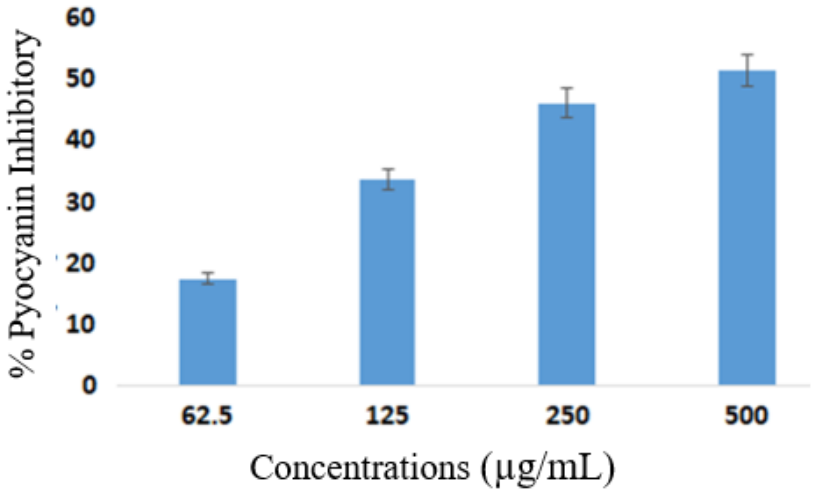


Figure 23: Pyocyanin inhibition assay of MeOH. P. aeruginosa was treated with various concentrations of MeOH (62.5-500 µg/mL). Dose-dependent inhibitory activity of MeOH was observed *in* the inhibition of Pyocyanin production (biosynthesis). Values are from three independent experiments ($P<0.05$).

CHAPTER FIVE

5. DISCUSSION

Medicinal plants are secondary metabolites. Plants continue to provide solutions to human and livestock ailments in traditional medicine systems for primary health care. The leaf of *A. polystachyus* is traditionally used for various medicinal purposes and has recently been shown to be effective in treating malaria and wounds (Demilew, *et al.,* 2018*;* Kifle, *et al.,* 2020). It also possesses antibacterial properties (Mailu, *et al.,* 2021). It has been observed and reported that the chemical constituents of plant species may vary depending on the environmental conditions, places where it grows, soil structure and so on (Moisa, *et al.,* 2019). Therefore, identification and analysis of phytochemical components aid in understanding of the involvement of natural chemical constituents and their mechanisms (Alawode, *et al.,* 2021). The variation in extracts could be explained by differences in plant parts, extraction solvents, and geographical location. In the current study, chemical compounds shown to have antimicrobial activities. These findings show that the detected phytochemical compounds indicate that the *A. polystachyus* is a valuable reservoir of bioactive compounds with significant medical value.

The qualitative analysis of MeOH based on biochemical assays indicated the presence of alkaloids, flavonoids, phenols, saponins, steroids, tannins, and terpenoids, respectively, however, glycosides were absent (Table 5). For instance, Demilew *et al*., (2018) reported that anthraquinones, flavonoids, glycosides, phenols, saponins, tannins, and terpenoids, are the most common phytochemicals that are found in leaves of *A. polystachyus*. According to (Maliu, *et al,*.2021) Preliminary qualitative phytochemical analysis made for *A. polystachyus* revealed the presence of alkaloid, flavonoids, glycosides, phenols, saponins, tannins, and terpenoids. The secondary metabolites in present study, reported that phytochemicals such as tannins, saponins, flavonoids, and alkaloids are bioactive compounds that have an extensive range of beneficial pharmacological effects like; anticancer, anti-diabetic, anti-inflammatory, antihypertensive, antimicrobial, and antioxidant activities, in addition to alleviating hypercholesterolemia (Mollova, *et al.,* 2023).

The compounds from GC-MS analysis of methanolic extract of *A. polystachyus* yield was found to be 20.5% and the yield from EO was recorded as 0.4% which agrees with the yields observed

from several different plant leaves. The UV spectra recorded in the range of 200-400 nm for EO indicated the presence of alkaloids, coumarins, flavonoids, phenols, and terpenes, respectively. Terpenes have a UV absorption maximum in the 210-220 nm range because of the conjugated pi systems; phenols have a UV absorption maximum in the 270-280 nm range due to the presence of aromatic rings; flavonoids have a UV absorption maximum in the 290-300 nm range due to hydroxyl groups; coumarins have a UV absorption maximum in the 310-320 nm range due to the presence of a coumarins ring; and alkaloids have a UV absorption maxima in the 330-340 nm range due to the presence of nitrogen atoms. The peak areas were used to calculate the individual compounds' percent occurrence (amount).

The compounds were identified based on the intensity of peaks that were generated in GC-MS chromatograms (Figure 17) and are summarized in Tables 7 (MeOH) and 6(EO) for the complete list of compounds. The peak areas were used to calculate the percent occurrence (amount) of the individual compounds. The major compounds found in MeOH were β-sitosterol acetate (16.06%), cholest-5-en-3-yl (9Z)-9-octadecenoate (9.54%), 1-dodecanol (7.57%), (S)-(E)-(-)-4-acetoxy-1-phenyl-2-dodecen-1-one (6.03%), Neophytadiene (5.7%), (E)-2-nonadecene (3.9%), hexanol-4-D2 (2.92%), and decane (2.4%). Several of these compounds are potentially bioactive. β-Sitosterol, a phytosterol present naturally in the cells and membranes of plants, has been shown to have antibacterial and antioxidant activities with various applications (Alawode, *et al.,*2021; Ododo, *et al.,* 2016; Pierre Luhata, *et al.,* 2021; Ravi, *et al.,* 2020); cholest-5-en-3-yl (9Z)-9-octadecenoate was found in the extracts that showed anticancer and antibacterial activities(Casillas-Vargas, *et al.,* 2021); 1-dodecanol is a long-chain fatty acid with high potential as antibacterial (MIC, 8 μg/mL) and antimycobacterial agents (Mukherjee, *et al.,* 2013; Togashi, *et al.,* 2007); Neophytadiene is a diterpenoids compound that possesses antimicrobial, anti-inflammatory, antibiofilm, and antioxidant properties (Raman, *et al.,* 2012; Mashamba, *et al.,* 2022); nonadecene, hexanol, and decane exhibit antibacterial activities (Ghavam, *et al.,* 2021; Ingram, *et al.,* 1980; Wang, *et al.,* 2011). Most of the major compounds were identified from the MeOH of *A. polystachyus* (seven out of eight), which has known bioactive functions. Thus, it may be expected that MeOH likely has the potential to exhibit bioactivity.

The major compounds detected by GC-MS in EO were Stearyl alcohol P721 (25.38%); cis-9-tetradecenoic acid, isobutyl ester (22.95%); butyl 9-tetradecenoate (10.62%); 11, 13-dimethyl-

12-tetradecen-1-ol acetate (10.14%); Ginsenol (3.48%); and diisooctyl phthalate @P1404 (2.54%). Stearyl alcohol has varied uses such as pharmaceutical dispensing, cosmetic creams, perfumery, antifoam agents, resins, surface active agents, and lubricants. Stearyl alcohol possesses antioxidant and antibacterial activities (Casillas-Vargas, *et al.,* 2021; Mukherjee, *et al.,* 2013). Cis-9 tetradecenoic acid has been reported for antibacterial, anti-inflammatory, and analgesic properties (Al-Marzoqi, *et al.,* 2016); butyl 9-tetradecenoate is reported for antibacterial activity (Ubaid, *et al.,* 2016); 11,13-dimethyl- 12-tetradecen-1-ol acetate is reported for the antimicrobial formulation of toothpaste (Oluwasina, *et al.,* 2023); ginsenol is a sesquiterpenoid that shows fungistatic action (Aleu, *et al.,* 2001); and diisooctyl phthalate is reported for antimicrobial and antioxidant activities (Abou El-Enain, *et al.,* 2023; Al-Marzoqi, *et al.,* 2016). It is noteworthy that the chemical composition of *A. polystachyus* EO is different from the other EOs. The monoterpenes were not detected, and one sesquiterpenoid (ginsenol) was present along with saturated fatty acids, fatty acid esters, alcohols, phenols, and benzoic acid ester. In a previous study, the EO of Acanthus ilicifolius (Acanthaceae) was reported to contain alkanes, fatty acids, benzoic acid esters, alcohols, and alkenes (Satyavani, *et al.,* 2015). Our result agrees with these results.

The antioxidation potential of MeOH and EO was tested by using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay that is based on the principle of reduction of DPPH by hydrogen donors to diphenyl picrylhydrazine. Both MeOH and EO were found to show an effective range of radical scavenging at varying concentrations (125-1000 µg/mL; µL/ml for EO). The activities of MeOH and EO ranged from 48.3 to 84.2 %RSA and 45.6 to 82 %RSA, respectively (Table 9) with dose-dependency. Ascorbic acid was used as a standard reference with the same concentrations and generated values of 90.3 – 96.7 %RSA. The activity of MeOH and EO was found to be comparable with the standard ascorbic acid at higher concentrations. This may be attributed to the presence of compounds found in this study possessing antioxidation properties such as Neophytadiene, β-sitosterol (MeOH) and Diisooctylphthalate (EO), respectively (Ododo, *et al.,* 2016; Raman, *et al.,* 2012; Al-Marzoqi, *et al.,* 2016). Several studies have reported the antioxidant property of natural products of plants. The antioxidation property is beneficial in scavenging free radicals involved in several diseases and metabolic pathways, and imparts hepatoprotective, immunoprotective, and neuroprotective effects (Ilyas, *et al.,* 2016).

The antimicrobial properties of extracts *A. polystachyus* against Gram positive and Gram negative the strength of the activity was directly proportional to the concentration of the extract loaded to the disk. The major compounds detected were shown resistance against commercial antibiotics compared with ZOI of *E. coli* (17.0 ± 0.57 mm) and other bacteria (19.0 ± 0.57- 21.67 ± 1.20 mm) (Figure 18 and Table 5). However, interestingly, we found higher inhibition (susceptibility) values for MeOH in *A. baumannii* (130.2%) and *P. aeruginosa* (100.3%) (Table 5). This may be due to decreased resistance or higher susceptibility against MeOH. Thus, it emphasizes the antibacterial potency of MeOH as a new, valuable, yet cost-effective measure to deal with antimicrobial-resistant bacteria, thereby eliminating infections. A moderate range of inhibition (34.2-59%) was observed for fungus, indicating antifungal activity of MeOH. The compounds listed in Table 3 from MeOH such as *β*-sitosterol, cholest-5-en-3-yl (9*Z*)-9-octadecenoate, 1-dodecanol, Neophytadiene, (*E*)-2 nonadecene, hexanol-4-D2, and decane possess antimicrobial properties (Ododo, *et al*., 2016; Wang, *et al*., 2011) that appear to be responsible for the observed inhibition of the bacterial and fungal species.

Among the Gram-positive strains, at 400 mg/mL, *S. aureus* showed higher inhibitory values (87.7%) than *E. faecalis* (63.16%) and *S. pyogenes* (67.7%). *S. aureus* is known to exhibit greater resistance to antimicrobials (Walesch, *et al.,* 2023); however, it showed higher susceptibility against MeOH here. An inhibition of 49% was observed in *E. coli* at 400 mg/mL; however, notably, higher inhibitory values were observed in the case of *P. aeruginosa* (100.3%) and *A. baumannii* (130.2%), which increased the inhibitory range. Gram-negative bacteria in general and *P. aeruginosa* and *A. baumannii*, in particular, have been known to exhibit higher resistance to antimicrobials and are categorized in the pathogen priority list (PPL) as "critical priority" by WHO (WOH, 2017). This is also evident from the lower ZOI of PC (gentamycin) in the case of *P. aeruginosa* (12.6 ± 0.33 mm) and *A. baumannii* (9.47 ± 0.56 mm), indicating higher resistance against commercial antibiotics compared with ZOI of *E. coli* (17.0 ± 0.57 mm) and other bacteria (19.0 ± 0.57- 21.67 ± 1.20 mm) (Figure 18 and Table 5). However, the inhibition intensity was less than that of MeOH (mentioned above). Among Gram-negative strains, *E. coli* was found to be the least susceptible (40.2-50%), and among Gram-positive, *S. aureus* showed the highest susceptibility (34-74%). The "critical priority" and "high priority" resistant strains, *A. baumannii*, *P. aeruginosa*, and *S. aureus* showed lesser resistance (or higher

susceptibility) in the ranges of 34-74, 68.7-89.8, and 52.2- 72.7%, respectively than other strains. These results are similar to MeOH and emphasizes the importance of EO as an effective antimicrobial.

The major compounds detected by GC-MS in EO summarized in (Table 9) indicate the presence of bioactive compounds such as stearyl alcohol, *cis*-9-tetradecenoic acid, isobutyl ester, butyl 9-tetradecenoate, 11,13-dimethyl-12- tetradecen-1-ol acetate, and diisooctylphthalate that demonstrated antibacterial activity and may be responsible for the achieved inhibition by EO (Al-Marzoqi, *et al*., 2016; Casillas-Vargas, *et al*., 2021; Mollova, *et al*., 2023). *C. albicans* exhibited an inhibition range of 33-53.7%. Ginsenol is reported for its fungistatic properties (Aleu, *et al.*, 2001; Satyavani, *et al*., 2015). Thus, MeOH and EO of *A. polystachyus* may be regarded as valuable for their potency against stringent and MDR bacterial strains, *S. aureus, A. baumannii*, and *P. aeruginosa*, in particular, and other pathogenic bacteria and fungus, in general, used in this study. In a previous report, an acetone extract of the capsule from *Eucalyptus camaldulensis* exhibited excellent antibacterial properties (140%) compared with controls against *A. baumannii* and *E. coli* and antifungal activity (96%) against *Rhizopus stolonifera* (Nars, *et al.*, 2019).

The MIC values of MeOH against strains varied from 0.5 to 1.0 mg/mL and MBCs/MFCs from 2 to 4 mg/mL. *E. coli* recorded an MIC of 1 mg/mL, which was higher than other bacteria, indicating higher resistance. Similar resistance patterns were observed in the antibacterial assay for *E. coli* (Figure 18 and Table 11). There was no other apparent difference observed among the strains, and it indicated the broad-spectrum activity of the extracts. Tolerance test values calculated as MBC/MIC (for bacteria) and MFC/MIC (for fungi) were between 4 and 8. The values of <4 indicate bactericidal activity, while a value of 8 indicates bacteriostatic action. The strains *A. baumannii*, *E. faecalis*, and *C. albicans* shown a value of 8, indicating bacteriostatic/fungistatic activity, while all others scored 4 indicating the bactericidal potential of MeOH and EO. In a previous report MeOH of *A. polystachyus*, MICs ranged from 100 to 200 mg/mL (for bacteria and *C. albicans*) and MBCs from 200 to 400 mg/ mL for bacteria (Mailu, *et al.*, 2021). These values are 100-400 times higher than those found in this study. This may be attributable to the cultivation, environmental, and geographical factors, including soil structure (Moisa, *et al.*, 2019). In another study, the acetone extract of *E. camaldulensis*, MIC values of

18-20 mg/mL were reported for *A. baumannii* and *E. coli* that showed a 140% inhibitory activity in the antibacterial assay. The values obtained in our study are lower than in this report (Nars, *et al.,* 2019). Several other plant species MeOH have been used to explore the antibacterial activity (Kim, *et al.,* 2020). The values observed are either comparable to or better than those reported.

The MIC values of EO for the tested microbes varied from 0.31 to 0.62 μL/mL and MBCs/MFCs from 1.25 to 2.5 μL/ mL. *P. aeruginosa* and *C. albicans* showed higher values (MIC of 0.62 μL/mL and MBC/MFC of 2.5 μL/mL), indicating higher resistance. All strains scored tolerance test values of 4, indicating EO‘s bactericidal/fungicidal activity. EO of several plant species have been investigated, and a wide range of MIC/ MBC/MFC have been reported (Mohammed, *et al.,* 2022; Galovićová, *et al.,* 2021). A recent report observed MICs of 50-100 μL/mL for some similar bacterial species used in this study and 6.25 μL/mL for *C. albicans*, which are much higher than found here (Mohammed, *et al.,* 2022). In another study, a wide range of MICs were reported from 0.2 to 12.5 μL/mL against bacterial and fungal strains (Galovićová, *et al.,* 2021). Our results are comparable to the lower range of this report. These results confirm the antimicrobial potential of the EO of *A. polystachyus* and may prove valuable against pathogenic and resistant microbial species.

Microbes develop resistance when challenged by conventional antimicrobials leading to impaired treatment of diseases. The resistance is most commonly associated with forming biofilms that protect microbes from surrounding environmental stresses, impedes phagocytosis, and confer the capacity for colonization and long-term persistence. Such ability is promoted by effective cell-to-cell communications (quorum sensing) within the microbial communities. As a result, highly structured biofilms can be formed, often identified in patients with chronic infections, such as chronic lung and wound infections. Therefore, exploring new sources with pharmaceutical properties that can interfere with, reduce, and eradicate biofilms for the effective treatment of such diseases is essential.

The MeOH and EO of *A. polystachyus* were investigated for their ability to reduce biofilm formation. The results are shown in Figure 21. Both MeOH and EO were effective and showed dose-dependent inhibition of biofilm formation, with MBIC values in the range of 0.5-1 mg/mL (MeOH) and 0.31-0.62 μL/mL, respectively. The Gram-negative representative bacteria were more resistant to the biofilm inhibitory effect of MeOH and EO than Gram-positive strains. At

the highest concentration, 1 mg/mL of MeOH, the biofilm inhibitory effect was in the following order: *E. faecalis* > *S. pyogenes* > *S. aureus* > *A. baumannii* > *E. coli* > *P. aeruginosa*. In the case of EO, the following order was observed: *S. pyogenes* > *E. faecalis* > *S. aureus* > *P. aeruginosa* > *E. coli* > *A. baumannii*. The reduction of the biofilm formation ability of microbes has been reported to vary across an extensive range (0.125-100 mg/mL). Our results agree with the reported MBIC values of MeOH of different plant species in the 0.25-1.0 mg/mL range (Alenazy, *et al.*, 2023; Perumal, *et al.*, 2013). Similarly, a wide range of MBICs for EO have been reported in the range of 2-8 µL/mL (Wang, *et al.*, 2020); and 50-100 μL/mL (Mohammed, *et al.*, 2022), respectively. Our results show better efficacy of EO with lower MBIC values.

Biofilm-forming MOs are difficult to treat with conventional antimicrobial agents. It can resist up to 1000-fold, thus increasing the cost of treatment. These microbial populations may further aggravate the AMR problem by transferring resistance genes to other populations (Bengtsson-Palme, *et al.*, 2018; De Oliveira, *et al.*, 2020). Therefore, targeting the inhibition of biofilm formation is a plausible direction to reduce and possibly eradicate biofilm-forming pathogens. Therefore, further extensive investigations are required to establish the effectiveness of antibiofilm extracts or compounds. Possible directions would be to isolate individual components of extracts and rigorously assess the potential of single compounds or synergy combinations.

Pyocyanin is a pigment distinctive to *P. aeruginosa* and plays a crucial role in infection and virulence. There is also a favorable relationship between pyocyanin and antibiotic resistance, thus empowering *P. aeruginosa* for AMR (Yang, *et al.*, 2020). The highest inhibition (51.6%) was at 500 μg/mL. Pejin *et al.* reported that at 0.5MIC (MIC, 19 μg/mL), phytol inhibited pyocyanin production by 51.94% (Pejin, *et al.*, 2015). Phytol was used as a purified single compound, and the 0.5MIC value (9.5 μg/mL) is much lower than our result (500 μg/mL), which contains a mixture of several compounds. However, in future studies, purification and identification of the individual compounds responsible for pyocyanin inhibition may be pursued. In another study, at 750 μg/mL, four different extracts of *Camellia nitidissima* inhibited pyocyanin production in the 51.25-67.5% range (Yang, *et al.*, 2020). Our results agree with those in this report. Thus, MeOH demonstrated pyocyanin inhibitory potential and showed promise to be further investigated.

CHAPTER SIX

6. CONCLUSIONS AND RECOMMENDATION

6.1. Conclusions

Plant natural products rich in phytochemicals are unfolding as possible solutions to combat pathogenic and resistant microbes. In this study, we explored *A. polystachyus* for its potential as an antioxidant and antimicrobial against selected bacterial and fungal species. The phytochemical composition of extracts by GC-MS revealed bioactive compounds. The antioxidant property was found in both MeOH and EO by the DPPH assay. The MeOH exhibited high inhibition for the "critical priority" and "high priority" strains, *A. baumannii*, *P. aeruginosa*, and *S. aureus* (87.75-130.20% inhibition). The EO also showed potential inhibition for the same strains in the range of 72.7-89.8%. The MIC (0.5-1 mg/mL; 0.31-0.62 μL/mL), MBC/MFC (2-4 mg/mL; 1.25-2.5 μL/mL), and MBIC (0.5-1 mg/mL; 0.31-0.62 μL/mL) values of MeOH and EO confirmed the inhibition potential as well as the inhibition of biofilm formation. Pyocyanin, an important factor in antibiotic resistance, virulence, and QS, was also inhibited by MeOH in the anti-QS assay using *P. aeruginosa* (51.6%, 0.5 mg/ mL). Thus, MeOH and EO extracted from *A. polystachyus* leaves have high potency and are valuable as antioxidants and antimicrobials. This study explored and established the phytochemical composition of *A. polystachyus* extracts and unveiled the potential antimicrobial properties that may prove useful to reduce the microbial population load and combat pathogenic and resistant microbes.

6.2. Recommendation

It is recommended to conduct further research to identify and isolate the active compounds present in the extracts of *A. polystachyus*. However, further characterizations are required to determine the components of the EO and MeOH of *A. polystachyus* using techniques such as high-performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR). Understanding the specific compounds responsible for the observed antibacterial, antifungal, antioxidant, antibiofilm, and quorum sensing inhibition activities can provide insights into their mechanisms of action and potential application in combating antibiotic-resistant bacteria. In

order to elucidate the mode of action of the extract, additional mechanistic studies should be conducted. Investigating the impact of the extract on microbial cell structure, biofilm formation quorum sensing pathways, and gene expression can provide a deeper understanding of how these natural compounds interact with bacterial systems.

Considering the rise of antibiotic resistance, it would be valuable to explore the potential synergistic effects of the extract with existing antibiotics. Combination studies can assess whether the extract enhance the efficacy of antibiotics or help to overcome resistance mechanism, potentially leading to more effective treatment options. Based on these recommendations, we can further we can further explore the potential of *A. polystachyus* extract as alternative therapeutic options, contribution to the fight against antibiotics resistance, and potentially improve patient outcomes in the treatment of bacterial infection.

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