

Phytochemical Screening and Antimicrobial Activity of *Agarista salicifolia* and
Phyllanthus reticulatus against Some Human Pathogens



By

Tekalign Thomas Tole

A Thesis Submitted to the Department of Applied Biology

School of Applied Natural Science

Presented in Partial Fulfillment of the Requirements for the Degree of Master of
Science in Applied Biology (Microbiology)

Office of Graduate Studies

Adama Science and Technology University

October, 2020
Adama, Ethiopia.

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Major Advisor: Dr. Teshome Geremew

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APPROVAL OF BOARD OF EXAMINERS

We, the undersigned, members of the Board of Examiners of the final open defense by Tekalign Thomas Tole have read and evaluated his Thesis entitled “**Phytochemical Screening and Antimicrobial Activity of *Agarista salicifolia* and *Phyllanthus reticulatus* against Some Human Pathogens**” and examined the candidate. This is, therefore, to certify that the Thesis has been accepted in partial fulfillment of the requirement of the Degree of Master of Science in Applied Biology with specialization in Applied Microbiology.

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To: Applied Biology Department

Subject: Thesis Submission

This is to certify that the Thesis entitled “**Phytochemical Screening and Antimicrobial Activity of *Agarista salicifolia* and *Phyllanthus reticulatus* against Some Human Pathogens**” submitted in partial fulfilment of the requirements for the degree of Master of Science in Applied Biology with specialization in Applied Microbiology of School of Applied Natural Science, M.Sc. program of Department of Applied Biology, Adama Science and Technology University and is a record of original research carried out by Tekalign Thomas Tole (ID No. PGR/18125/11), under our supervision, and no part of the Thesis has been submitted for any other degree or diploma in any other University. Therefore, we recommend that the student has fulfilled the requirements and hence hereby he can submit the Thesis to the Department.

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LIST OF ACRONYMS AND ABBREVIATIONS

ATCC	American type culture collection
CSA	Central Statistical Authority
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO	Dimethyl Sulfoxide
EPS	Extracellular polymeric substances
GIT	Gastrointestinal tract
ICU	Intensive Care Units
MIC	Minimum Inhibitory Concentration
NMR	Nuclear Magnetic Resonance
TLC	Thin Layer Chromatography

ABSTRACT

This study aimed to evaluate *in vitro* antimicrobial activities of *Agarista salicifolia* and *Phyllanthus reticulatus* plants extracted by different solvents against five human pathogens. Antimicrobial activity of plant extracts was carried out by agar disk diffusion, minimum inhibitory concentration (MIC), anti-biofilm activity, anti-quorum sensing assay, and bioautography for purified compound. Phytochemical screening tests were carried out following standard protocols and the result revealed that steroids, tannins, saponins and flavonoids were present in the ethanol extract of leaves of *Agarista salicifolia*. The leaves and roots of *Agarista salicifolia* and *Phyllanthus reticulatus*, respectively, were extracted with ethanol and afforded 7.5% and 6.3 % yield, respectively. Antimicrobial activities of ethanol leave extracts of *Agarista salicifolia* showed maximum activity against *Staphylococcus aureus* (25.3 ± 1.2 mm) and minimum activity against *Candida albicans* (11 ± 1.52 mm). The ethanol root extract of *Phyllanthus reticulatus* showed maximum activity against *Staphylococcus aureus* (17.7 ± 1.20 mm) and minimum activity against *Pseudomonas aeruginosa* (10.7 ± 2.92 mm). A relatively low MIC value was recorded for the leave extract of *Agarista salicifolia* (25mg/ml) against the tested pathogens. The highest anti-biofilm activity was shown by *Agarista salicifolia* ethanol leaves extract on *Salmonella typhimurim* through test tube method. Highest anti-swarming activities were shown by *Agarista salicifolia* leave extract at 50mg/ml on *Salmonella typhimurim*, *Pseudomonas aeruginosa* and *Candida albicans*. Anti-quorum sensing activity by *Agarista salicifolia* leaves extract against *Staphylococcus aureus*, *Streptococcus pyogen* and *Salmonella typhimurium* at 5mg/ml. Bioautography test displayed that compound 1 inhibited the growth of *Pseudomonas aeruginosa* better than the *Staphylococcus aureus*. Silica gel column chromatography to leaves extract of *Agarista salicifolia* afforded one acylaminosugars derivative characterized as N-acetyl chitobiose derivative. The overall results of the present study revealed that *Agarista salicifolia* leave extract has high antimicrobial, anti-biofilm and anti-quorum sensing activity against the test microorganisms thus; *Agarista salicifolia* is a better source of antimicrobial agent hence can be of interest in the development of new chemotherapeutic drug.

Keywords: antibacterial activity, plant extract, phytochemical screening, Pathogens and Spectrum.

CHAPTER 1

INTRODUCTION

1. Background

Plants have been used from ancient times to cure diseases, to relieve physical suffering and also utilized as sources of spices, dyes, poisons and drugs. Ancient peoples all had acquired some knowledge of medicinal plants. Oftentimes these primitive attempts at medicine were based on superstition and speculation. The chemical compounds responsible for antimicrobial activities are often the secondary metabolites of plants or natural products as they are usually referred to. The World Health Organization (WHO) defines traditional medicine as health practices, knowledge and beliefs applied single or in combination to prevent illnesses and maintain well-being (WHO, 2011).

Plant derived medicines afford benefits such as profound therapeutic benefits, less or no side effects, low cost, and easy accessibility (Selvamohan et al., 2012). However, some medicinal herbs for some reasons have not found broader use and sometimes are referred as 'disremembered plants' taking into account the growing demand for natural ingredients that might be used as food additives, preventing plant, animal and human diseases. It is rational to revise the 'forgotten plants' by evaluating their applicability and profits using new scientific study methods (Abdel Rahman et al., 2011). In general, antimicrobial screening of medicinal plants offer the chance of discovering active candidates with novel molecules of unique structure and potency.

The increase of microbial resistance to currently available antibiotics has demanded the need to search for new antibacterial agents. Gram positive bacteria such as *Staphylococcus aureus*, *Streptococcus pyogenes* are mainly responsible for post-operative wound infections, toxic shock syndrome, endocarditis, osteomyelitis and food poisoning (Benayache et al., 2001). Gram negative bacterium such as *Pseudomonas aeruginosa* is present in human intestine and causes lower urinary tract infection, coleocystis or septicaemia (Benhassaini et al., 2003). *Candida albicans* is part of our normal microflora that commonly lives in or on our bodies. It

can be found in the gastrointestinal tract, the mouth, and the vagina. *Candida species* are the most common cause of fungal urinary tract infections (Lundstrom and Sobel, 2001).

Diverse antibiotics use their inhibitory activity on different pathogenic organisms (Chanda and Rakholiya, 2011). Drug resistance in human pathogenic microorganisms has been developed due to miss use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases. The development of antibiotic resistance is multifactorial, including the specific nature of the relationship of microbes to antibiotics, the usage of antimicrobial agent, host characteristics and environmental factors. This condition has forced scientists to search for new antimicrobial substances from various sources as new antimicrobial chemotherapeutic agents, but the cost for production of synthetic drugs is high and they produce adverse effects compared to plant derived drugs (Abiramasundari et al., 2011).

Ethiopia has a large diversity of plant species containing many useful bioactive constituent (Tadeg et al., 2005). Traditionally in rural Ethiopia, *Phyllanthus reticulatus* and *Agarista salicifolia* are used to treat many kinds of diseases such as gastrointestinal infections, insecticides, wound, dermatitis and skin infections. However, these two plants (*Phyllanthus reticulatus* and *Agarista salicifolia*) were not subjected to a comprehensive phytochemical screening so far. This study tried to fill the knowledge gap and will offer a better understanding of the phytoconstituents for their various actions. With this background information, the present study aimed at elucidating the in vitro antimicrobial activity of *Phyllanthus reticulatus* and *Agarista salicifolia* on selected test pathogenic bacterial and fungal species, namely: *Staphylococcus aureus*, *Streptococcus pyogens*, *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Candida albicans* by using different solvent extraction methods.

1.2. OBJECTIVES

1.2.1. General Objective

To perform phytochemical screening and evaluate antimicrobial activity of the crude extracts and fractionated extract for *Phyllanthus reticulatus* and *Agarista salicifolia*.

1.2.2. Specific Objectives

- To extract crude compound from root parts of *P. reticulatus* and leave parts of *A. salicifolia*.
- To perform phytochemical screening tests against crude extracts of *Phyllanthus reticulatus* and *Agarista salicifolia*.
- To evaluate the antimicrobial activity of the crude extracts against five selected human pathogens (*Staphylococcus aureus*, *Streptococcus pyogens*, *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Candida albicans*) by disk diffusion assay.
- To identify secondary metabolites from the crude extracts of *A. salicifolia* leaves parts.
- To characterize fractionated compound using 1D NMR.
- To evaluate antimicrobial activities of crude extracts and fractionated compounds against human microbial pathogens.

CHAPTER 2

LITERATURE REVIEW

2.1. Traditional Medicinal Plants

Floras as a source of medicinal compounds have continued to play a dominant role in the maintenance of human health since ancient times. According to the World Health Organization plant extracts or their active constituents are used as folk medicine in traditional therapies of 80% of the world's population. Over 50% of all modern clinical drugs are of natural product origin (Kirbag et al., 2009). Phytochemicals such as vitamins (A, C, E and K), carotenoids, terpenoids, flavonoids, polyphenols, alkaloids, tannins, saponins, pigments, enzymes and minerals that have antimicrobial and antioxidant activity (Madhuri and Pandey, 2009).

The precise function of many phytochemicals is still unclear; however, a substantial number of studies have shown that they are involved in the interaction of plants, pests or diseases. Antimicrobial screening of plant extracts and phytochemicals, then, represents a starting point for antimicrobial drug discovery. Phytochemical studies have attracted the attention of plant scientists due to the development of new and sophisticated techniques. These techniques played a significant role in the search for additional resources of raw material for pharmaceutical industry (Shakeri et al., 2012). The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments.

In the last few years, a number of studies have been conducted in different countries to prove such efficiency. Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant (Nascimento et al., 2000). In Ethiopia, there are plentiful medicinal plants described for handling of various diseases. Herbal medicine is considered vital part of the Ethiopian culture and plays a key and crucial role in the public healthcare (Birhan et al., 2011; Tolossa et al., 2013). One thousand identified medicinal plant species are stated in the Ethiopian Flora, however, many others are not yet known.

About 300 of these species are repeatedly mentioned in various sources. The greater concentration of medicinal plants are found in the south and south western Ethiopian parts of the country following the concentration of natural and cultural variety (Edwards, 2001).

2.1.1. *Agarista salicifolia* as Medicinal Plants and Its Chemical Properties

Agarista salicifolia belongs to the family Ericaceae. *A. salicifolia* is a shrub or tree about 10-20 m tall with branched stems and leaf buds of reddish color. The leaves are prominent midrib, very dense at the top of twigs, young leaves are reddish. The fruit capsules are small seeds dispersed by wind (Bosser et al., 1981).



Figure 1. Leaves of *Agarista salicifolia* (Source: Nganou et al., 2018).

Traditionally a leaf of *A. salicifolia* is applied on the wounds caused by scarification acting analgesic properties. In East Africa, the plant is also used as an insecticide and as an antidote against the poison arrows. The bark is mixed with fresh water and then drunk as an aid to digestion or after excessive meat. The roots and bark of the of *A. salicifolia* are used for the treatment of several diseases in Central Africa for example when bites by snakes, skin diseases, added to baitto kill (Moshi et al., 2007). In Ethiopia, grilled plant is pulverized and applied to ulcerous wounds. It is also used to treat snake bites and varies skin infections. Externally, the leaves are also used for the treatment against scabies. The leaves are fatally toxic to humans and livestock, even the leaves can cause vomiting, seizures, difficulty in breathing and coma. The roots are also toxic (Loriaux et al., 1973).

2.1.2. *Phyllanthus reticulatus* as Medicinal Plants and Its Chemical Properties

Phyllanthus reticulatus (Family Euphorbiaceae) commonly known as pancoli or karineli, (Synonym: *Kirganelia reticulata* Poir) is a large glabrous or pubescent shrub with smooth or lenticellate branches growing from 8-10 ft in height, 0.7-3 cm wide, produced on short lateral branchlets, looking like leaflets of a compound leaf (Kathriarachchi et al., 2006).



Figure 2. Roots of *Phyllanthus reticulatus* (Source; Shalini Sharma and Sunil Kumar, 2013).

In history this plant has been used in medicine, *P. reticulata* used in treated of chronic cough chronic diarrhoea, cryptococcal meningitis, Herpes zoster, oral candidiasis, oral sores, skin infections, skin rashes, tuberculosis (Kisangau et al., 2011). *P. reticulata* elaborates different class of organic compounds of, medicinal importance including alkaloids, flavonoids lignans, sitosterol, polyphenols, triterpenoids, saponins, coumarins phytosterols (Sheng et al., 2010). The maximum number of phytochemical compound is present in leaves then stem and root (Venkata and Narasimhudu, 2012). The leaves and bark are sweet and cooling and hence are used in constipation and urinary disorder while leaves juice is used for the treatment of diarrhoea in children (Ghani, 2003).

The roots and bark is used as astringent, diuretic and alternative (Nadkarni, 1976). For bleeding gums, pill of this drug along with camphor and cubebs is kept in the mouth (Kirtikar and Basu, 1980).

Table 1. Uses of the selected plant species in local medicine (Information were collected from traditional practitioners by Tekalign Thomas on 06/02/2020).

Local name	Scientific name	Common medicinal uses
Xayro (Gofigna)	<i>Phyllanthus reticulatus</i>	Tongue disease, Anti-diarrheal, Varies skin infections, Tooth infections, Headache, Cough, Anti-parasitic, Stomach disease.
Canqo (Gofigna)	<i>Agarista salicifolia</i>	Uses as insecticidal, Skin infections, Rat killers, Eye Disease, Snakes bite, Ear infection, Treatment of edema.

Table 2. Botanical data of the investigated plants in literature (Sankannavar and Patil, 2012).

Scientific name	Plant parts	Solvent	Antimicrobial activity	References
<i>Phyllanthus reticulatus</i>	leaves	Ethanol and Methanol.	<i>Staphylococcus aureus</i> , <i>Salmonella typhi</i> , <i>Vibrio cholera</i> and <i>Pseudomonas aeruginosa</i>	Sankannavar and Patil, 2012.
	Roots	Chloroform, Methanol and Hexane.	<i>Escherichia coli</i> , <i>Pseudomonas aeruginosa</i> , <i>Salmonella typhi</i> and <i>Staphylococcus aureus</i>	Shruthi et al., 2010.
<i>Agarista salicifolia</i>	Leaves	Methanol	<i>Escherichia coli</i> , <i>Staphylococcus aureus</i> , <i>Salmonella typhi</i> and <i>Salmonella typhi</i>	Getahun and Masresha, 2010.
	Roots	Methanol	<i>Pseudomonas aeruginosa</i> , <i>Escherichia coli</i> and <i>Streptococcus pyogenes</i>	Kang et al., 2011.

2.2. Feature of Test Microorganisms

Microorganisms used in the study were *Staphylococcus aureus*, *Streptococcus pyogenes*, *Salmonella typhimurium*, *Pseudomonas aeruginosa* and pathogenic fungi, *Candida albicans*.

Staphylococcus aureus were members of the genus *Staphylococcus* (staphylococci) are Gram-positive cocci that tend to be organized in grape-like clusters (Ryan and Ray, 2004).

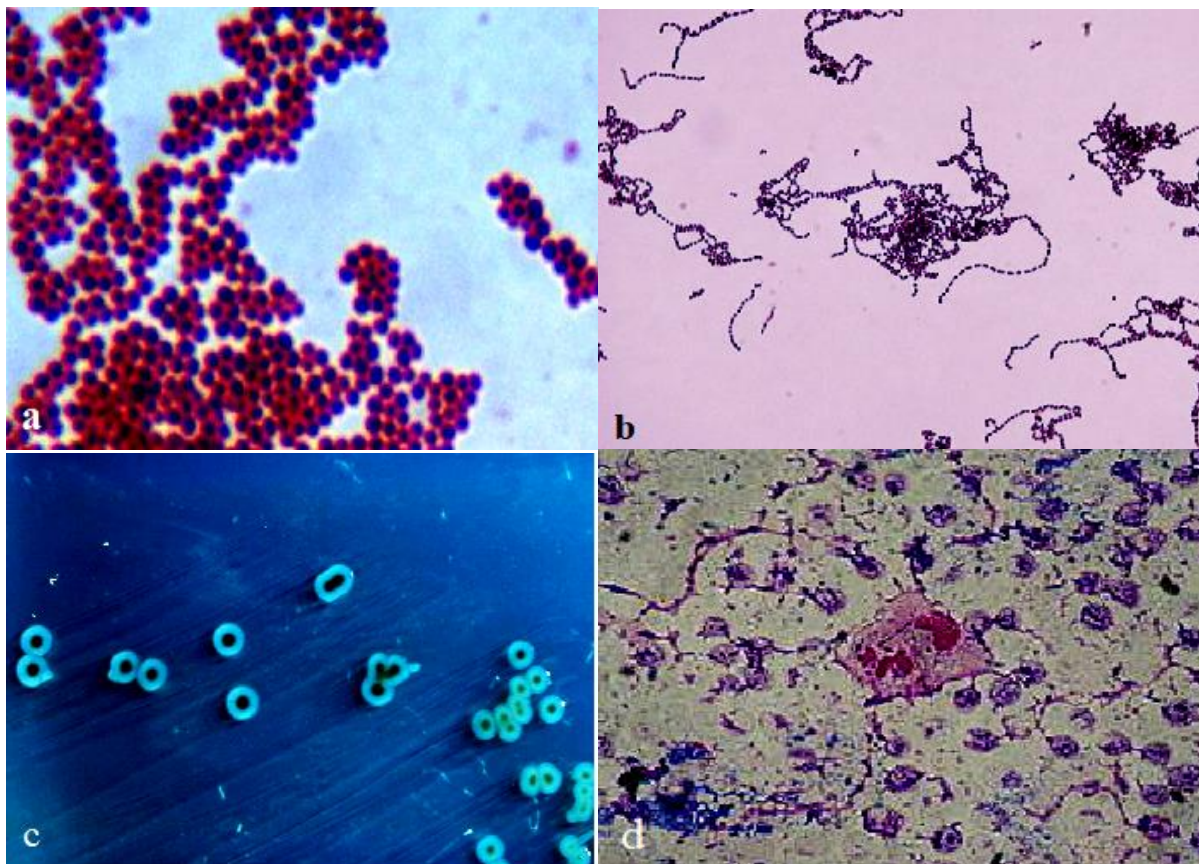


Figure 3. Potential human pathogens so far reported for antimicrobial activity tests. (a) Gram stain of *S. aureus* cells (Colco, 2005). (b) Gram stain of *S. pyogenes* cells (Pier et al., 2004). (c) Gram stain of *S. typhimurium* cells (Vågene et al., 2018) and (d) Gram stain of *P. aeruginosa* cells (Balcht and Smith 1994).

Staphylococcus aureus is a facultative anaerobe that grows at an optimum temperature of 37°C and an optimum pH of 7.5. *Staphylococci* are spherical cells about 1µm in diameter organized in asymmetrical clusters. Single cocci, pairs, tetrads, and chains are also seen in liquid cultures (Figure 3a). Young cocci stain strongly gram-positive; on aging, many cells

become gram-negative. *Staphylococci* are non-motile and do not form spores (Brooks et al, 2007). On nutrient agar, following aerobic incubation for 24 hours at 37°C, colonies are 1 – 3mm in diameter, have a smooth glistening surface, an entire edge and an opaque pigmented appearance. In most strains, pigmentation is golden with orange, yellow and cream varieties. On MacConkey agar, colonies are small to medium in size and pink or pink-orange in color (Mackie and McCartney, 1989). *S. aureus* creates white colonies that tend to turn a buff-golden color with time, which is the basis of the species epithet aureus (golden). Most, but not all, strains show a rim of clear β -hemolysis surrounding the colony (Ryan and Ray, 2004)

Staphylococci are enormously popular colonizers of humans and animals. They live mainly on the skin, predominantly in moist areas such as the anterior nares (nose), axilla and groin. Between one-third and three-quarters of individuals carry these organisms at any one time. Staphylococcal infections occurs worldwide and recently emerging hyper virulent or multi resistant strains spread fast over wide geographical areas. The bacteria survive in the air, on objects or in dust for days; thus they can contaminate environments (such as hospitals) and continue to be transmitted over long periods of time. Some individuals may shed the organism more heavily than others. Staphylococcal infections are acquired from either self (endogenous) or external (exogenous) sources (Irving et al., 2006).

Staphylococcus aureus causes severe infections of the skin, soft tissues, bone, lung, heart, brain or blood (Irving et al., 2006). Include pneumonia, bacteremia leading to secondary pneumonia and endocarditis, osteomyelitis secondary to bacteremia and septic arthritis, seen in children and in patients with a history of rheumatoid arthritis. Diseases caused by Staphylococcal toxins include scalded skin syndrome and toxic shock syndrome (Sherris, 1984). Resistance to penicillin G can be predicted by a positive test for β -lactamase; approximately 90% of *S. aureus* produce β -lactamase. Resistance to nafcillin (and oxacillin and methicillin) occurs in about 35% of *S. aureus* and approximately 75% of *S. epidermidis* isolates (Brooks et al., 2007). Alternative antibiotics for resistant organisms (e.g. MRSA) include vancomycin, erythromycin and gentamicin. Some strains become resistant to multiple antibiotics (Irving et al., 2006).

Streptococcus pyogenes is a species of Gram-positive, aerotolerant bacterium in the genus *Streptococcus*. *Streptococci* are round bacteria (Figure 3b). The species name is derived from

Greek words meaning 'a chain' (streptos) of berries (coccus) (Latinized from kokkos) and pus (pyo) forming (genes), because streptococcal cells tend to link in chains of round cells (see image) and a number of infections caused by the bacterium produce pus. The main criterion for differentiation between *Staphylococcus* spp. and *Streptococcus* spp. is the catalase test. *Staphylococci* are catalase positive whereas streptococci are catalase negative (Ryan and Ray, 2004). *S. pyogenes* can be cultured on fresh blood agar plates under ideal conditions; it has an incubation period of 1 to 3 days (Aziz et al., 2010).

Streptococcus pyogenes (Figure 3b) typically inhabits the throat, genital mucosa, rectum, and skin. Of healthy individuals, 1% to 5% has throat, vaginal, or rectal carriage. In healthy children, such carriage rate varies from 2% to 17%. There are four methods for the transmission of this bacterium: inhalation of respiratory droplets, skin contact, and contact with objects, surface, or dust that is contaminated with bacteria or, less commonly, transmission through food. Such bacteria can cause a variety of diseases such as streptococcal pharyngitis, rheumatic fever, rheumatic heart disease, and scarlet fever. The number of pharyngitis cases is higher in children when compared with adults due to exposures in schools, nurseries, and as a consequence of lower host immunity. Such cases Streptococcal pharyngitis occurs more frequently from December to April (later winter to early spring) in seasonal countries, possibly due to changing climate, behavioral changes or predisposing viral infection. Disease cases are the lowest during autumn (Androulla et al., 2016).

Streptococcus pyogenes is the cause of many human diseases, ranging from mild superficial skin infections to life-threatening systemic diseases (Ryan and Ray, 2004). Infections typically begin in the throat or skin. The most striking sign is a strawberry-like rash. Examples of mild *Streptococcus pyogenes* infections include pharyngitis (strep throat) and localized skin infection (impetigo). Erysipelas and cellulitis are characterized by multiplication and lateral spread of *S. pyogenes* in deep layers of the skin. The bacterium is found in neonatal infections (Baucells et al., 2015).

Salmonella typhimurium is a genus of rod-shaped (bacillus) Gram-negative, non-spore-forming bacteria of the family *Enterobacteriaceae* (Figure 3c).

Salmonella typhimurium are rod-shaped (bacillus), non-spore-forming, predominantly motile *enterobacteria* with cell diameters between about 0.7 and 1.5 μm , lengths from 2 to 5 μm , and peritrichous flagella (Fàbrega and Vila, 2013). When grown on ordinary agar, the organism forms spherical smooth colonies. They are chemotrophs, obtaining their energy from oxidation and reduction reactions using organic sources. They are also facultative anaerobes, capable of generating ATP with oxygen ("aerobically") when it is available, or when oxygen is not available, using other electron acceptors or fermentation ("anaerobically") (Fàbrega and Vila, 2013).

Most subspecies of *Salmonella* produce hydrogen sulfide (Clark and Barret, 1987). Which can readily be detected by growing them on media containing ferrous sulfate, such as is used in the triple sugar iron test. RVS broth can be used to enrich for *Salmonella* species for detection in a clinical sample (Snyder and Atlas, 2006). *S. typhimurium* can also be detected and subtyped using multiplex (Alvarez et al., 2004), or real-time polymerase chain reactions (PCR) from extracted *S. typhimurium* DNA (Hoorfar et al., 2000).

In nature, *Salmonella typhimurium* (Figure 3c) may survive several years in the soil and infamously known for causing food poisoning in humans, resulting in gastroenteritis in humans and other mammals. Food poisoning often occurs when a person consumes uncooked meat or comes in contact with the fecal matter of an infected individual. The most common symptoms include: diarrhea, vomiting, and fever; this lasts between two to five days. *S. typhimurium* infects the host by penetrating the intestinal mucosa and migrating to the spleen and liver where it causes systematic disease (Rosenberger et al., 2000).

Salmonella typhimurium is known to be able to cause back pain or spondylosis. It can manifest as five clinical patterns: gastrointestinal tract infection, enteric fever, bacteremia, local infection, and the chronic reservoir state (Figure 3c). The initial symptoms are nonspecific fever, weakness, and myalgia among others. In the bacteremia state, it can spread to any parts of the body and this induces localized infection or it forms abscesses. The forms of localized *Salmonella* infections are arthritis, urinary tract infection, infection of the central nervous system, bone infection, soft tissue infection (Choi et al., 2010). Infection may remain as the latent form for a long time, and when the function of reticular endothelial cells is deteriorated, it may become activated and consequently, it may secondarily induce spreading

infection in the bone several months or several years after acute salmonellosis (Choi et al., 2010).

Pseudomonas aeruginosa is a common opportunist pathogen belonging to the genus *Pseudomonas* (Mackie and McCartney, 1989). *Pseudomonas aeruginosa* is obligate aerobe, motile, rod-shaped and measuring about 0.6 x 2 µm. It is gram-negative and occurs as single bacteria, in pairs, and occasionally in short chains (Figure 3d). Some times producing a sweet or grape-like or corn taco-like odor (Brooks et al., 2007). Its production of blue, yellow, or rust-colored pigments distinguishes it from most other Gram-negative bacteria. The blue pigment, pyocyanin, is produced only by *P. aeruginosa*. Fluorescin, a yellow pigment that fluoresces under ultraviolet light is by *P. aeruginosa* and other free-living less pathogenic *Pseudomonas* species. Pyocyanin produced and fluorescin combined produce a bright green color that diffuses throughout the medium (Ryan and Ray, 2004). *Pseudomonas aeruginosa* grows well at 37–42 °C; its growth at 42 °C helps differentiate it from other *Pseudomonas* species. It does not ferment carbohydrates, but many strains oxidize glucose (Brooks et al., 2007). *P. aeruginosa* commonly live soil, water, and vegetation and can be isolated from the skin, throat, and stool of healthy persons. They commonly inhabit hospital food, sinks, taps, mops and respiratory equipment.

Spread is from patient to patient through contact with fomites or by ingestion of contaminated food and water (Baron, 1996). *Pseudomonas aeruginosa* causes infections in healthy individuals and those who are hospitalized or have a compromised immune system as a result of other diseases. A variety of human infections are frequently associated with this bacterium: Urinary tract infections, Ventilator-associated pneumonia, surgical site infections, Ear infections (external otitis, malignant external otitis), Skin and soft tissue infections, including hot tub folliculitis, and osteomyelitis. Individuals with compromising conditions, such as HIV/AIDS, cystic fibrosis, chemotherapy-related neutropenia, and diabetes have an increased risk of getting an infection and developing complications (Trautmann et al., 2008). *Pseudomonas aeruginosa* is frequently resistant to many commonly used antibiotics. Although many strains are susceptible to gentamicin, tobramycin, colistin, and amikacin, resistant forms have developed, making susceptibility testing essential (Baron, 1996).

Candida albicans is opportunistic pathogenic yeast (Gow, 2017). That is a common member of the human gut flora and belonging to the genus *Candida*.

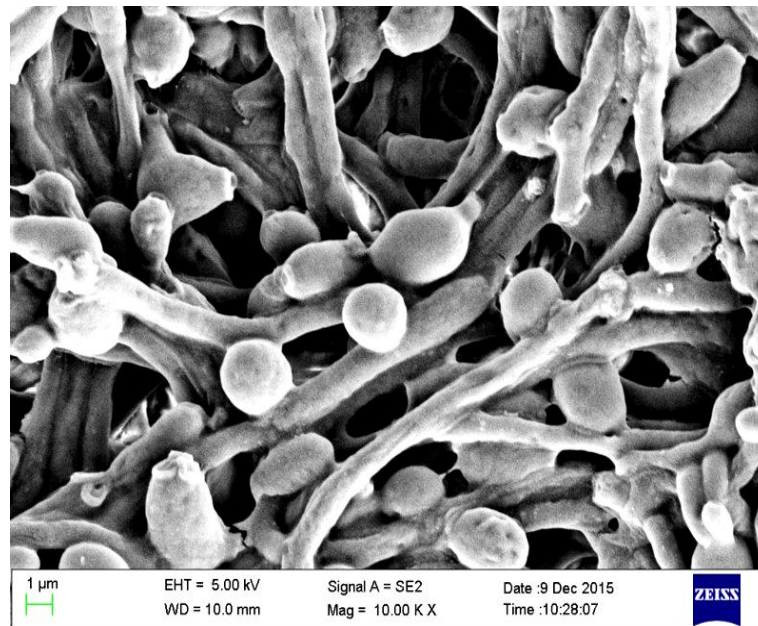


Figure 4. *C. albicans* visualized using scanning electron microscopy (Source; Barnett, 2004).

Candida albicans is frequently used as a typical organism for fungal pathogens (Kabir et al., 2012). It is commonly mentioned to as a dimorphic fungus since it grows both as yeast and filamentous cells. However, it has several diverse morphological phenotypes (Kadosh, 2019). *C. albicans* was for a long time considered an obligate diploid organism without a haploid stage. This is, however, not the case. Next to a haploid stage *C. albicans* can also exist in a tetraploid stage. The latter is formed when diploid *C. albicans* (Figure 4) cells mate when they are in the opaque form (Hickman et al., 2016). The diploid genome size is approximately 29 Mb, and up to 70% of the protein coding genes have not yet been characterized (Kabir et al., 2012). *C. albicans* is simply cultured in the lab and can be studied both in vivo and in vitro. Depending on the media different studies can be done as the media influences the morphological state of *C. albicans*. A special type of medium is CHROM agar *Candida*, which can be used to identify different species of candida (Sevilla and Odds, 1986).

Candida albicans is opportunistic pathogenic yeast that is a common member of the human gut flora (Gow, 2017). It can also survive outside the human body (James et al., 2019). It is identified in the gastrointestinal tract and mouth in 40–60% of healthy adults (Kerawala and Newlands, 2010). It is usually a commensal organism, but it can become pathogenic in immune compromised individuals under a variety of conditions (Erdogan and Rao, 2015). It is one of the few species of the genus *Candida* that causes the human infection candidiasis, which results from an overgrowth of the fungus (Erdogan and Rao, 2015).

Candidiasis is for example often observed in HIV-infected patients (Calderone and Clancy, 2012). *C. albicans* is the most common fungal species isolated from biofilms either formed on (permanent) implanted medical devices or on human tissue (Kumamoto, 2002). *C. albicans*, *C. tropicalis*, *C. parapsilosis*, and *C. glabrata* (Figure 4) are together responsible for 50–90% of all cases of candidiasis in humans (Martins et al., 2014). A mortality rate of 40% has been informed for patients with systemic candidiasis due to *C. albicans* (Singh et al., 2017). However, these numbers may not truly reflect the true extent of damage this organism causes, given new studies showing that *C. albicans* can cross the blood brain barrier (Wu et al., 2019).

Candida albicans infections are the top cause of fungal infections in critically ill or otherwise immune compromised patients (Sydnor and Emily, 2011). *Candidas* are classified as one of the most common groups of organisms that cause hospital-acquired infections. Mainly high-risk individuals are patients that have recently undergone surgery, a transplant or are in the Intensive Care Units (ICU) (Brosnahan and Mandy, 2013). These patients mostly develop oropharyngeal or thrush candidiasis, which can lead to malnutrition and interfere with the absorption of medication (Sardi, 2016). Parts of the body that are usually infected include the skin, genitals, throat, mouth, and blood (Tortora et al., 2010).

2.3. Biofilm

A biofilm encompasses any syntrophic consortium of microorganisms in which cells stick to each other and often also to a surface (Lopez et al., 2010). These adherent cells become surrounded within a slimy extracellular matrix that is composed of extracellular polymeric substances (EPS) (Hall et al., 2004). The cells within the biofilm produce the EPS components, which are typically a polymeric conglomeration of extracellular polysaccharides, proteins, lipids and DNA (Aggarwal et al., 2016). Because they have three dimensional arrangements

and signify a community lifestyle for microorganisms, they have been metaphorically described as "cities for microbes"(Watnick and Kolter, 2000). Biofilms may form on living or non-living surfaces and can be prevalent in natural, industrial, and hospital settings (Lear and lewis, 2012). The microbial cells developing in a biofilm are physiologically distinct from planktonic cells of the same organism, which, by contrast, are single-cells that may float or swim in a liquid medium (Toole and Kolter, 1998).

Biofilms are not just bacterial slime layers but biological systems; the bacteria form themselves into organized functional community. Biofilms can attach to a surface such as a tooth, rock, or surface, and may include a single species or a diverse group of microorganisms. The biofilm bacteria can share nutrients and are sheltered from harmful factors in the environment, such as desiccation, antibiotics, and a host body's immune system. A biofilm regularly begins to form when a free-swimming bacterium attaches to a surface, Christine and Berdell, (tenth edition).

2.3.1. Origin and Formation of Biofilm

Biofilms are assumed to have arisen during primitive Earth as a defense mechanism for prokaryotes at that time, as the conditions of primitive earth were too harsh for the survival of prokaryotes. Biofilms protect prokaryotic cells by providing them homeostasis encouraging the development of complex interactions between the biofilm cells (Hall et al., 2004).

2.3.2. Formation of Biofilms

The development of a biofilm initiates with the attachment of free-floating microorganisms to a surface (Watnick and Kolter, 2000). The first colonist bacteria of a biofilm may adhere to the surface primarily by the weak van der Waals forces and hydrophobic effects (Brindet et al., 2001). If the colonists are not immediately separated from the surface, they can anchor themselves more forever using cell adhesion structures such as pili.

The development of a biofilm may permit for cumulative cell colony (Figure 5) (or colonies) to be increasingly resistant to antibiotics. Cell to cell communication or quorum sensing has been shown to be involved in the formation of biofilm in several bacterial species (Brindet et al., 2001). The process is summarized by five major stages of biofilm development.

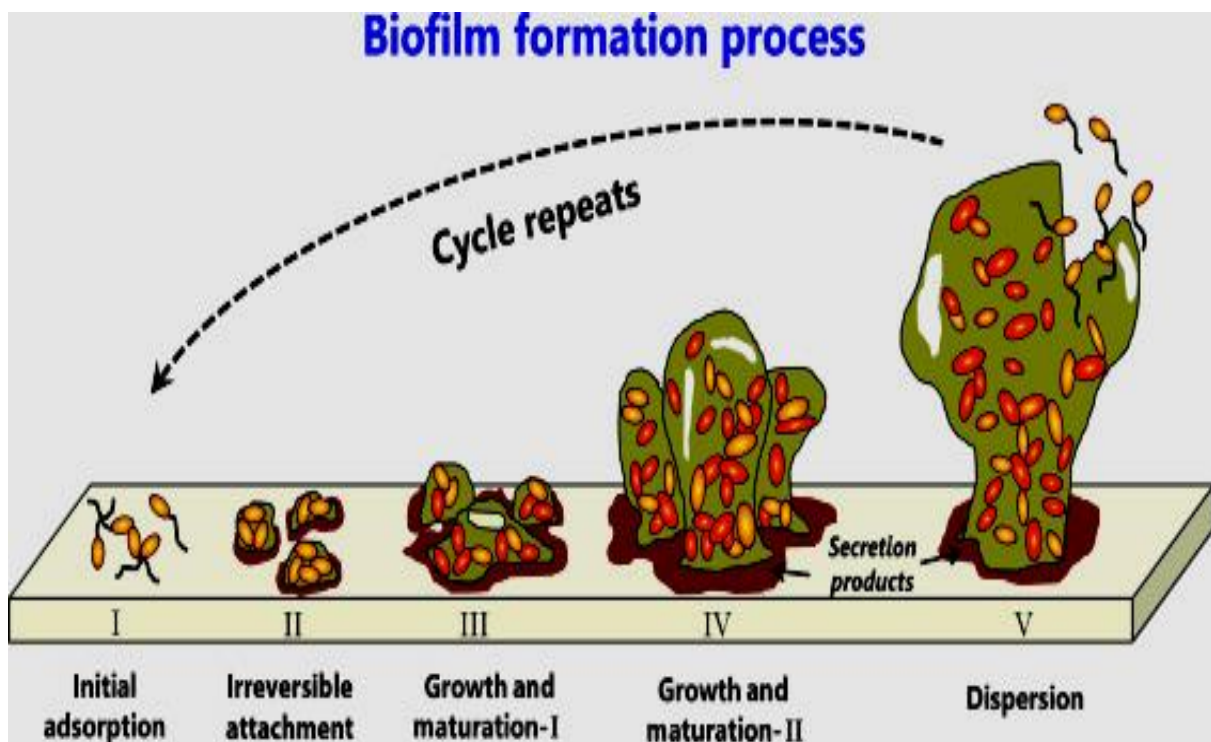


Figure 5. Biofilms are a communal of one or more types of microorganisms that can grow on many different surfaces area. These organisms are mostly belonging to bacteria, unicellular fungi, and protists. These microorganisms are also able to form dental plaque, a slimy buildup of bacteria that forms on the surfaces of teeth. It indicates a major stage of biofilm development (Fu et al., 2017).

2.3.3. Quorum Sensing

In biofilm formation many species of bacteria are able to communicate with one another through a mechanism called quorum sensing (Naves, 2010). During quorum sensing signaling molecules attach to receptors of new bacteria and help in transcription of genes within a single species of bacteria as well as between different bacterial species. QS system allows communication between intra species and interspecies which involves in terms of biofilm formation, food shortages and environmental stress conditions, such as disinfectants, antibiotics, bacterial colonization, the identification of annoying species, the establishment of normal intestinal flora as well as the prevention of harmful intestinal flora (Miller and Basseler, 2001).

2.3.4. Biofilm Forming Bacteria and Fungi

Approximately all (99.9%) of microorganisms have the capacity to form biofilm on a wide range of surfaces i.e. biological and inert surfaces (Sekhar, 2009). Biofilm forming capability has been reported in large number of bacterial species such as *P. aeruginosa*, *S. epidermidis*, *E. coli* spp, *S. aureus*, *E. cloacae*, *K. pneumoniae* (Parsek and Singh). Different fungi are capable of biofilm growth among fungi; different *Candida* species are the most common colonizers of implanted medical device (Kumamoto and Ramage, 2005). *Fusarium* species and *Malessezia pachydermatis* also form drug resistant biofilms on implanted medical devices (Martinez and Fries, 2010). It has been also studied that the biofilm formation (Figure 5) of microorganisms shown different stages (Fu et al., 2017).

2.4. Secondary Metabolites of Some Traditional Medicinal Plants

It was experimentally proved that plants are the sustainable source of diverse bioactive metabolites that can provide lead structures for development of novel drugs (Hossainzaded et al., 2015). Now-a-days, the plant preparations successfully used in the treatment of many infectious diseases are envisaged for antimicrobial potency. However, their antimicrobial efficacy ascribed to the existence of diverse array of bioactive principles. During the past few decades, over 500,000 bioactive metabolites have been isolated from diverse terrestrial plants and many of them are utilized to design and develop new therapeutic agents in pharmaceutical firm(Mendelson et al., 1995).

2.4.1. Secondary Metabolites of *Agarista salicifolia*

Previous phytochemical investigations on some species of the genus *Agarista* led to the isolation of triterpene and flavonoids (quercetin-3-O- α arabino furanoside, épicatechine, quercetine-3-O- β glucurono pyranoside (Burkill, H M. 1994). The work of Lhuilier in 2007 showed a comparative study by LC-MS composition of flavonoid fractions of *A. salicifolia* and *A. polyphylla*. This resulted in the identification "on line" say that is to without isolation 8 compounds of type glycosylate flavonols: quercetin-3-Ogalactoside, quercetin-3-O-glucoside, quercetin-3-Orhamnoside, genin quercetin, kaempferol-3-Oarabinopyranoside, kaempferol-3-O-arabinofuranoside, kaempferol-3-O-rhamnoside and kaempferol-3-Oglucuronide (Figure 6) (Nganou et al., 2018).

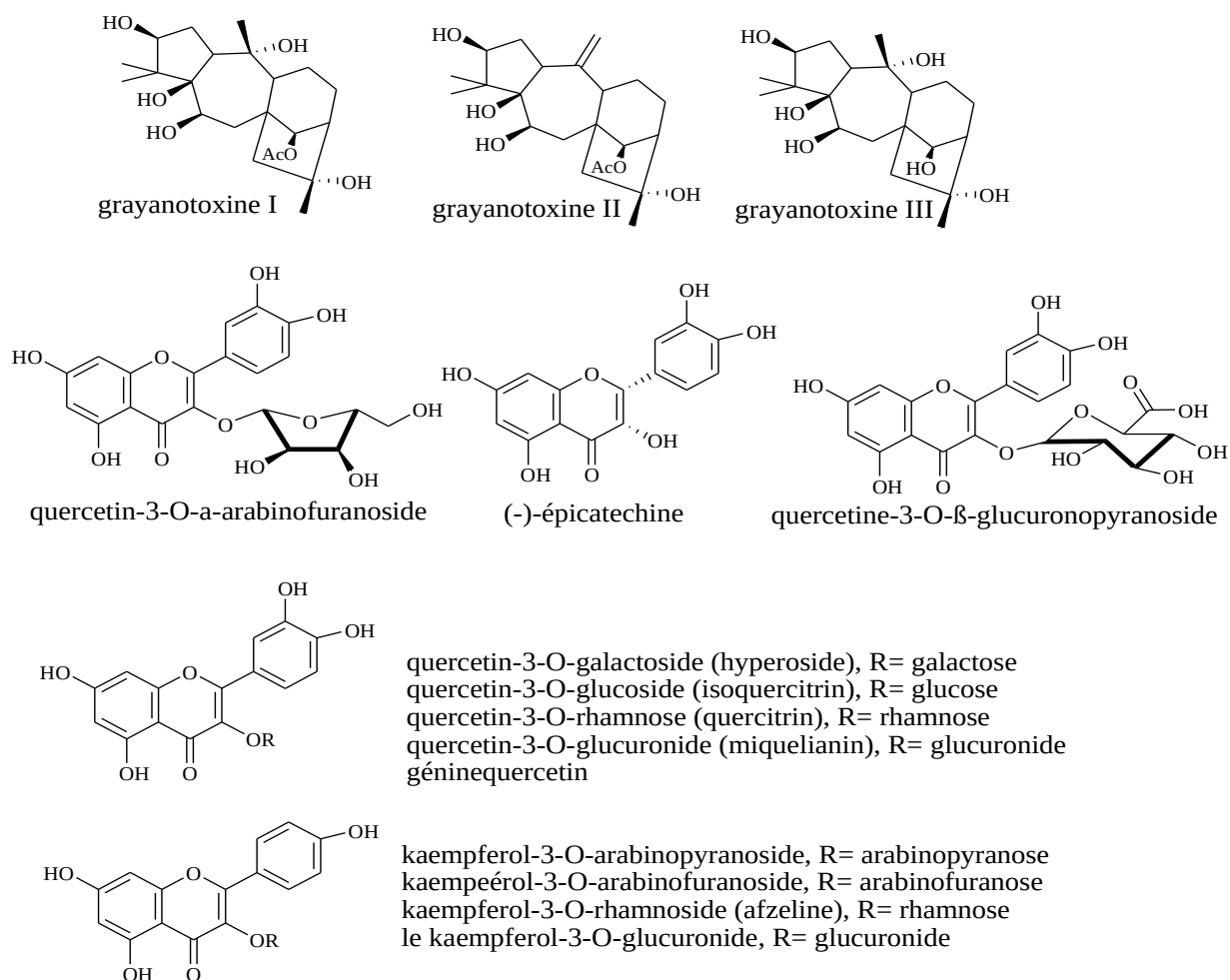


Figure 6. Compounds isolated from the genus *Agarista* (Nganou et al., 2018).

2.4.2. Secondary Metabolites of *Phyllanthus reticulatus*

Phytochemical investigations on *Phyllanthus reticulatus* led to the isolation of three main compounds (lupeol, lupeol acetate, and stigmasterol) (Jamal et al., 2008). Other isolated compounds are friedelin, epifriedelinol, betulin, beta-sitosterol, betulinic acid. Coumarin was isolated from stem bark (Begum et al., 2006). Eight compounds (β - sitosterol-3-O- β -glucoside, stigmasterol- 3- O- β - glucoside, methyl gallate, ellagic acid, kaempferol, astragalin, including two flavonoid glycoside rutin (quercetin- 3- rutinoside) and quercetin-3-O- β - D glucopyranoside (isoquercitrin) were isolated from the butanol soluble fraction of the methanolic extract of the leaves of *phyllanthus reticulatus* by conventional methods (Lam et al., 2007) (Figure 7).

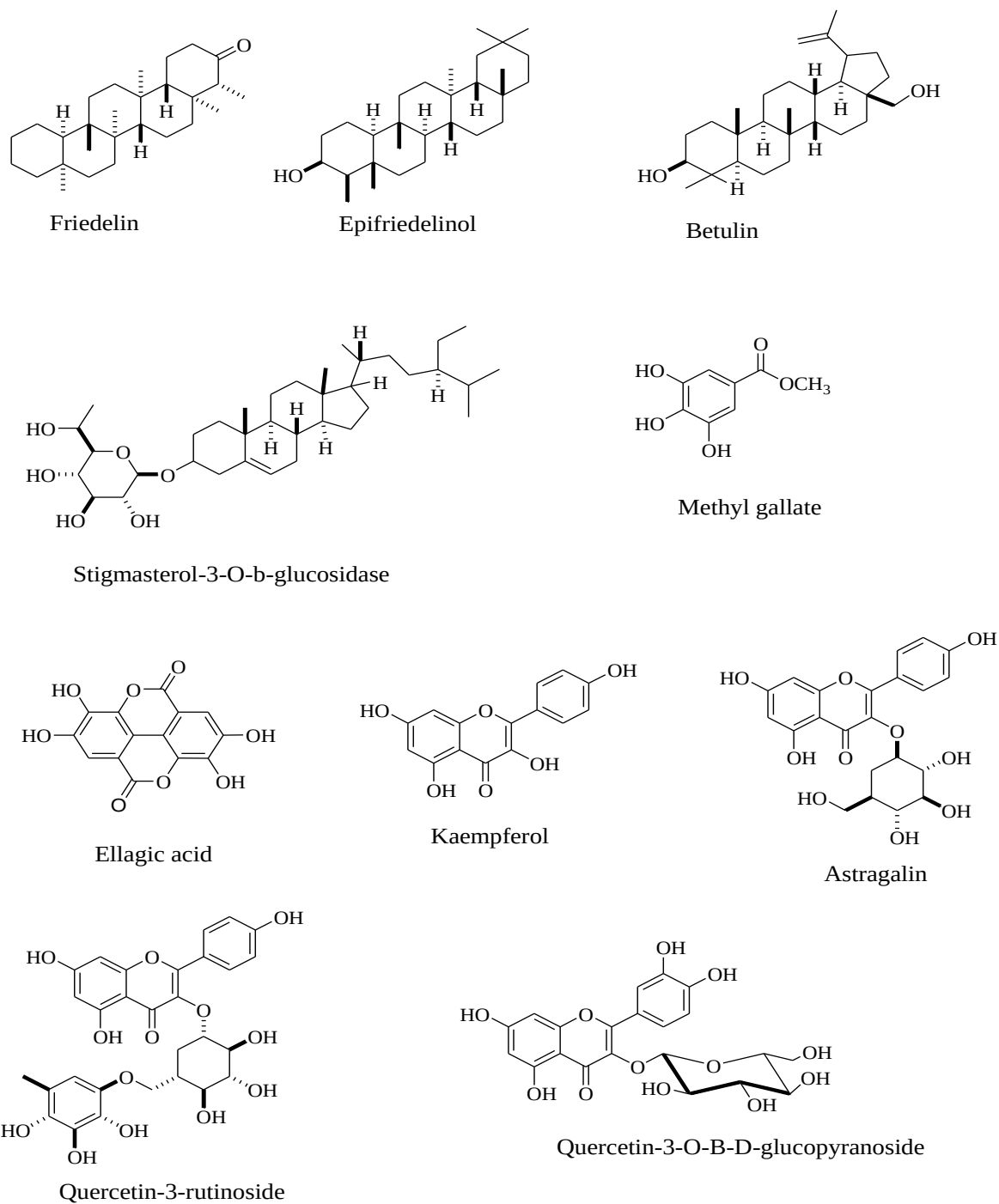


Figure 7. Structures of Phytoconstituents of *phyllanthus reticulatus* (Source; Shalini Sharma and Sunil Kumar, 2013).

Rutin was also quantitated from the methanolic leaf extract by thin layer high performance chromatography (Gajera et al., 2009). Ellagic acid was found as the chemical constituent for the inhibition of rheumatoid arthritis (Shruthi and Ramachandra, 2011). The structures of some chemical constituents have been shown in Figure 10.

2.5. Phytochemical Constituents of *Agarista salicifolia*

Phytochemical constituents of *A. salicifolia* shown the presence of tannins, alkaloids, cardiac glycosides, anthocyanin, steroids, polyphenols and terpenoids, these compounds play a major role to protect animals and human being against diseases (Haile Hosana L et al., 2015).

2.6. Phytochemical Constituents of *Phyllanthus reticulatus*

Phytochemical study of *Phyllanthus* species reported the occurrence of terpenoids, alkaloids, glycosides, flavonoids, tannins and saponins (Lee et al., 2016). Phenolic compounds, especially tannins, are the major constituents of *Phyllanthus* plants. More than 100 phenolic constituents with diverse biological activities were comprehensively identified in the fruits of *P. emblica* using HPLC-MS. It is also emphasized that different parts of *Phyllanthus* plants have different isomers of the same compounds. *Phyllanthus* species are rich in phytochemical diversity, with compounds such as tannins, phenylpropanoids, terpenoids, phenolic compounds, flavonoids, alkaloids, saponins and many of their glycosides (Yang et al., 2012).

2.7. Techniques Used for Characterisations of Bioactive Molecules from Plants

Purification and isolation of bioactive compounds from plants is a technique that has undergone new development in recent years (Altemimi et al., 2015). This modern technique offers the ability to parallel the development and availability of many advanced bioassays on the one hand, and provided precise techniques of isolation, separation, and purification on the other. The goal when searching for bioactive compounds is to find an appropriate method that can screen the source material for bioactivity such as antioxidant, antibacterial, or cytotoxicity, combined with simplicity, specificity, and speed (Yang et al., 2012).

2.7.1. Structural Clarification of the Bioactive Molecules

Determination of the structure of certain molecules uses data from a wide range of spectroscopic techniques such as UV-visible, Infrared (IR), Nuclear Magnetic Resonance (NMR), and mass spectroscopy. The basic principle of spectroscopy is passing electromagnetic radiation through an organic molecule that absorbs some of the radiation, but not all. By measuring the amount of absorption of electromagnetic radiation, a spectrum can be produced. The spectra are specific to certain bonds in a molecule.

Depending on these spectra, the structure of the organic molecule can be identified. Scientists mainly use spectra produced from either three or four regions-Ultraviolet (UV), Visible, Infrared (IR), radio frequency, and electron beam for structural clarification (Popova et al., 2009).

2.7.1.1. UV-Visible Spectroscopy

UV-visible spectroscopy can be performed for qualitative analysis and for identification of certain classes of compounds in both pure and biological mixtures. Preferentially, UV-visible spectroscopy can be used for quantitative analysis because aromatic molecules are powerful chromophores in the UV range. Natural compounds can be determined by using UV-visible spectroscopy (Kemp, 1991). The UV-Vis spectroscopy was used to determine the total phenolic extract (280 nm), flavones (320 nm), phenolic acids (360 nm), and the total anthocyanins (520 nm). This technique is not time-consuming, and presents reduced cost compared to other techniques (Urbano, 2006).

2.7.1.2. Infrared Spectroscopy

Some of the frequencies will be absorbed when infrared light passes through a sample of an organic compound; however, some frequencies will be transmitted through the sample without any absorption occurring. Infrared absorption is related to the vibrational changes that happen inside a molecule when it is exposed to infrared radiation. Therefore, infrared spectroscopy can essentially be described as a vibrational spectroscopy. Different bonds (C–C, C=C, C≡C, C–O, C=O, O–H, and N–H) have diverse vibrational frequencies. If these kinds of bonds are present in an organic molecule, they can be identified by detecting the characteristic frequency absorption band in the infrared spectrum (Urbano, 2006).

2.7.1.3. Nuclear Magnetic Resonance Spectroscopy

(NMR) NMR is primarily related to the magnetic properties of certain atomic nuclei; notably the nucleus of the hydrogen atom, the proton, the carbon, and an isotope of carbon. Moreover, it will demonstrate which atoms are present in neighboring groups. Ultimately, it can conclude how many atoms are present in each of these environments (Kemp, 1991).

2.8. Antimicrobial Activities of Traditional Medicinal Plants and Its Mechanisms of Actions Against Microbial Cells

Traditional medicinal plants possess various chemical substances that support certain physiological and biochemical activities in the human body and they are known as phytochemicals. These chemicals are non-nutritive substances used to heal various infectious diseases, as well as provide disease preventive properties (Mickymaray et al., 2016). With advances in phytochemical practices, numerous active principles have been isolated from medicinal plants and presented as a valuable drug in contemporary systems of medicine. Mostly, the pharmacological activity of medicinal plants resides in their secondary metabolites, which are relatively smaller in quantity in contrast to the primary molecules such as carbohydrates, proteins, and lipids.

Plant secondary metabolites are commonly accountable for their antimicrobial properties (Pandian et al., 2006). These metabolites offer clues to manufacture new structural types of antimicrobial and antifungal chemicals that are comparatively safe to humans (Pandian et al., 2006). Plant antimicrobial secondary metabolites are generally categorized into three broad classes, namely phenolic compounds, terpenes, and alkaloids (Ganesan et al., 2017). These compounds have numerous mechanisms that underlie antimicrobial activity, e.g., disturbing microbial membranes, weakening cellular metabolism, control biofilm formation, inhibiting bacterial capsule production, attenuating bacterial virulence by controlling quorum-sensing, and reducing microbial toxin production (Ganesan et al., 2017).

2.9. Natural Anti-biofilm and Anti-quorum Sensing Agents with Clear-Cut Mechanisms

Many plant derived natural products possessed antimicrobial and anti-biofilm functions in vitro. A variety of molecules derived from natural plants or medicinal herbs extract as well as the underlying mechanisms in anti-biofilm function were identified. The anti-biofilm effects of natural products are mainly relying on the following aspects, the inhibition of formation of polymer matrix, suppression of cell adhesion and attachment, interrupting extra cellular matrix (ECM) generation and decreasing virulence factors production, thereby blocking quorum sensing (QS) network and biofilm development (Lu et al., 2019).

CHAPTER 3

MATERIALS AND METHODS

3.1. Plant Sample Collection

The plant materials used in this study comprised leave of *Agarista salicifolia* and root of *Phyllanthus reticulatus*. These plants collected from Sawla town located in Gamo Gofa zone of the Southern Nations, Nationalities and Peoples Region. This town has a latitude and longitude of 6°18'N 36°53'E with an elevation of 1,395 meters above sea level (Figure 8). The area is characterized by hills and elevations with varied landscapes that resulted for the presence of distinct climatic and different natural flora resources that contributes for good agronomic improvement. The region is dominantly plateau comprising, mountains and with valleys. Plants comprise tree and bushes with smaller extents and dispersed trees. Soil nature is dominantly clay with compact regions. The region characterized variety rainy seasons. (CSA, 2007). Collected fresh sample were packed in plastic bags and transported into the laboratory of Adama Science and Technology University in the month of January 2020. The plant materials were authenticated by a botanist at Addis Ababa University.

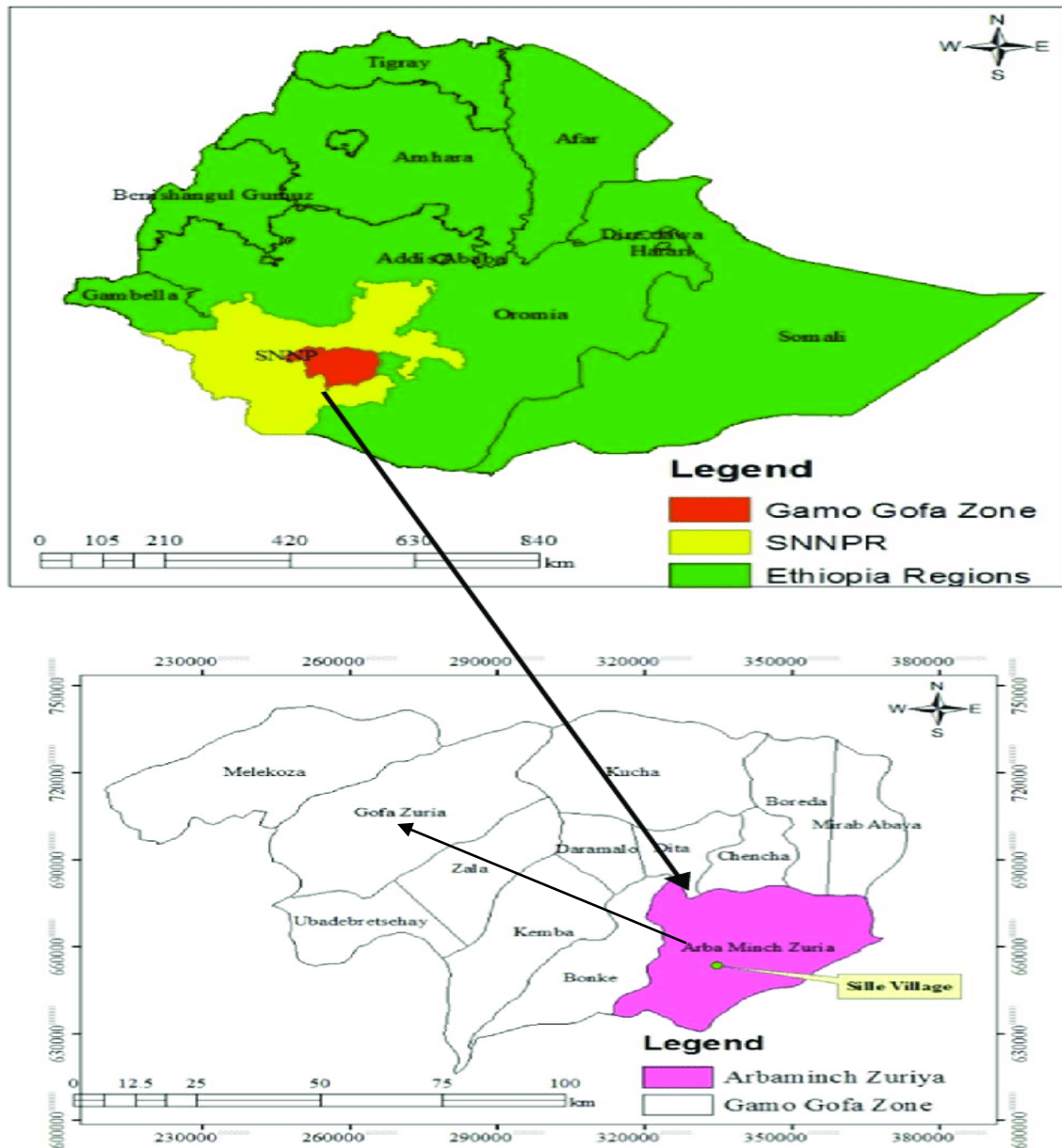


Figure 8. Map of study area; location of Gofa Zuriya in Gamo Gofa Zone, south-west Ethiopia. (Source; CSA, 2007)

3.2. Study Design

This study involved a laboratory based experimental design. The antimicrobial activities of the plant extracts against the test pathogens were determined using disk diffusion method. The minimum inhibitory concentrations (MIC) were determined for extracts against the selected pathogens using the broth dilution method. Anti-biofilm activity was tested using test tube and swarming motility test methods. Anti-quorum sensing was assessed using disk diffusion assay. Bioautography procedure was used to determine the number of antimicrobial compounds separated by thin layer chromatography plates (TLC). Screenings of the plants were performed using standard methods and isolation of bioactive compounds was done using spectroscopic techniques (NMR). The treatments included two plants with one part each extracted by three solvents (acetone, ethanol and water) and each extract applied on five test pathogens in three replications. Gentamicin and ketoconazole (i.e. alternative antibiotics for resistant strain) were used as positive controls for bacterial and fungal pathogens respectively, whereas Dimethyl Sulphoxide (DMSO) was used as a negative control for both. The study was carried out in Applied Biology laboratory of Adama Science and Technology University.

3.3. Test Pathogens

Microorganisms used in this study represent pathogenic species. Consists of bacterial strains (*Staphylococcus aureus* ATCC 25923), (*Streptococcus pyogenes* ATCC 11778), (*Salmonella typhimurium* ATCC 13312), (*Pseudomonas aeruginosa* ATCC 27823) and fungal strain, (*Candida albicans* ATCC 10231). Standard bacterial and fungus cultures were obtained from Adama Regional laboratory, and were maintained on Mueller-Hinton Broth (MHB) at 37°C and Sabouraud Dextrose agar (SDB) at 30°C respectively for further experiments.

3.4. Culture Media and Chemicals

Mueller-Hinton Agar (MHA), Sabouraud Dextrose agar (SDA), Nutrient broth and potato dextrose broth. Also acetone, ethanol and water were used for extraction process. These media and the solvent were purchased from companies.

3.5. Preparation of Plant Extract

Air dried and powdered plant materials (10g) were extracted by maceration with 100ml of acetone, ethanol (97%) and water at room temperature. The extracts were then filtered and concentrated under vacuum in rotary evaporator to give (as percentage of powdered plant materials). All the extracts were kept in tightly stopped bottle in refrigerator until used for antimicrobial testing.

All the crude extracts were weighed and the percentage yields of the crude extracts were recorded using the following formula.

$$\text{Percentage yield} = \frac{x_1 - x_2}{y} \times 100\%$$

Where, x_1 = Weight of container with crude extract, x_2 = Empty container and y = the weight of the soaked powder (Parekh and Chanda, 2007).

3.6. Preparation of Plant Extracts Standard Concentrations

Each aqueous, ethanol and acetone extract (1g) was taken and the aqueous extract was dissolved in 10ml sterile distilled water, while acetone and ethanol extracts were dissolved separately in 10ml of Dimethyl Sulphoxide (DMSO). Thus 100mg/ml of stock was obtained as a standard concentration of aqueous, ethanol and acetone extracts (Almola, 2010). The antibiotics were reconstituted by dissolving 20mg of powder (Gentamicin) in 2ml Dimethyl Sulphoxide and 10mg (ketoconazole) of powder in 1ml Dimethyl Sulphoxide to get a concentration of 10mg/ml of each of the antibiotics.

3.7. Preparation of Inoculum

Bacterial cultures grown in the bacteriological Mueller-Hinton Broth (MHB) at 37°C was adjusted at 1.5×10^8 colony forming units (CFU/ml) and fungal cultures grown in Sabouraud Dextrose Broth (SDB) at 30°C was adjusted at 2ml with a concentration of 10^6 spores/ml.

3.8. Preparation of McFarland Standard

McFarland number 0.5 standard was prepared by mixing 9.95ml 1% H_2SO_4 in distilled water and 0.05ml 1% BaCl_2 in distilled water in order to estimate bacterial concentration (Saeed and Tariq, 2007). The preparation was kept in flask and used for contrast of bacterial suspension.

3.9. Plant Extracts Activity Assay

3.9.1 Paper Disk Diffusion Assay

Two hundred microliter of a culture suspension of testing microorganisms (the turbidity was adjusted to approximately 1.5×10^8 CFU/ml) were spread on Muller Hinton Agar (MHA) medium for bacteria and 2ml of a culture suspension with a concentration of 10^6 spores/ml was added to Sabouraud Dextrose agar (SDA) for fungus. 100mg/ml of plant extract soaked filter paper discs (6mm in diameter) was placed on the agar plates which was inoculated with the tested microorganisms. Gentamicin (10mg/ml) and ketoconazole (10mg/ml) disk was used as the positive control for bacteria and fungus respectively, and 10% Dimethyl Sulphoxide-soaked filter paper disk was used as the negative control for each. The plates were subsequently incubated at 37°C and 30°C for 24h respectively. After incubation the growth inhibition zone (zones showing no microbial growth) was quantified by measuring the diameter of the zone of inhibition in mm (Kumar et al., 2009).

3.9.2. Determination of Minimum Inhibitory Concentration (MIC)

3.9.2.1. MIC against Test Pathogens

Plants extracts that showed antimicrobial activity with disc diffusion were selected for determination of MIC using broth dilution method. Two fold serial dilutions were used to dilute the initial concentration of the plant extract (100mg/ml) by transferring 1ml from the plant extract (stock) solution into 1ml of sterile nutrient broth kept in a flask and mixing the contents thoroughly and then serially diluting in 3 other flasks. The serial dilutions gave concentrations of 100mg/ml, 50mg/ml, 25mg/ml, and 12.5mg/ml (Hadia et al., 2012). After obtaining different concentrations of the extract, each concentration was inoculated with bacterial cultures (1ml) grown overnight were adjusted to 0.5McFarland standard, equivalent to 1.5×10^8 CFU/ml (*Staphylococcus aureus*), (*Streptococcus pyogenes*), (*Salmonella typhimurium*), (*Pseudomonas aeruginosa*) and with 1ml of the standardized fungal spore suspension (*Candida albicans*) and incubated at 37°C for 24h and 30°C for 72h respectively (Hadia et al., 2012). The growth of the bacterial and fungal test pathogens was assessed by comparing turbidity or cloudiness of broth containing test tubes with the negative control (broth lacking plant extracts). The lowest concentration, at which there was no turbidity

(determined by naked eye), was considered as the Minimum Inhibitory Concentration (MIC) value of the extract (Abou-Elkhair et al., 2010; Radojević et al., 2012).

3.9.3. Anti-biofilm Assay

3.9.3.1. Test tube Method

Test organisms (1ml) obtained by adjusting turbidity to 0.5McFarland standards (1.5×10^8 CFU/ml) was transferred to test tubes containing 10ml lysogenic broth. Twenty milligram per milliliter (20mg/ml) and thirty milligram per milliliter (30mg/ml) of ethanol extracts of leaves of *A. salicifolia* were added separately to the test tubes containing the test organisms and incubated at 37°C for 72 hours. The medium was removed, and the tubes were washed with distilled water, air dried and biofilm formation in the tubes was tested using crystal violet (to make clearly visible). All tests were carried out in triplicates and results of the test were recorded.

3.9.3.2. Swarming Motility Assay

Swarm plates were prepared using nutrient agar containing 0.5% peptone, 0.2% yeast extract, 50mg/ml of plant extracts and 1.0% glucose per 500ml of distilled water. Twenty microliters of standardized inoculums of microorganisms were placed on the center of the plate. Similar volume of test organism was placed on the center as negative control plate (agar without extract) and the plates were incubated for overnight at 37°C upright position for 24h (Kuchma et al., 2015).

3.9.4. Bioassay for Anti-Quorum Sensing

3.9.4.1. Preliminary Screening (Disk diffusion assay)

To assess the anti-quorum sensing ability of *A. salicifolia* leaves extracts the qualitative disc diffusion methods was used. Fifty microliters of bacterial culture was plated on lysogenic broth agar. Sterile paper discs (Whatman No.1, 6mm diameter) with *A. salicifolia* leaves extract (5mg/ml) were plotted above the agar. The Petridish were incubated at 37°C for 24h. Results were determined by observing the growth inhibition colorless ring diameter; opaque circles around the extract loaded paper discs and around positive controls (Gentamicin

10mg/ml) loaded discs. The negative control was broth loaded paper discs (Ramim et al., 2015).

3.9.5. Thin Layer Chromatography (TLC) Analysis of the Extracts

Two solvent systems with differing polarities were used to analyze 1mg/ml of the *A. salicifolia* leaves extract placed in a band of 1cm by thin layer chromatography. These solvent were n-hexane: ethyl acetate (8:2). Visible bands were marked under ultraviolet light (254 nm and 360 nm wavelengths).

3.9.6. Qualitative Antibacterial Activity Assay by Bioautography

Thin layer chromatography plates was prepared and developed in the two solvent systems, hexane: ethyl acetate (8:2) dried overnight under a stream of air to remove residual solvent, which might inhibit bacterial growth. The plate was sprayed with two bacterial cultures *S. aureus* and *P. aeruginosa*; 10ml of a dense fresh bacterial culture was centrifuged at 4000rpm for 15 min to concentrate the bacteria. The supernatant was discarded and the combined pellets re-suspended in 5ml of fresh Müller-Hinton broth. The plate was sprayed with the concentrated suspension until they were just wet, dried in air to remove excess liquid and incubated overnight at 37°C at 100% relative humidity. After incubation, plate was sprayed with a 2mg/ml solution of purple-violet (Sigma chemicals). Observation of clear zones on the chromatograms indicates inhibition of growth by separated antimicrobial compounds after incubating for about 2h at 37°C under 100% relative humidity (Begue and Kline, 1972).

3.9.7. Qualitative Tests: phytochemical screening

The concentrated filtrates from the ethanol leaves extracts of *A. salicifolia* was used to detect for the presence of secondary plant metabolites including, steroids, tannins, alkaloids saponins, flavonoids and terpenoids using standard methods (Trease, 1989; Christen, 2000; Young and Woodside, 2001; MacNee, 2005).

3.9.7.1. Test for Steroids (Lieberman-Burchard test)

A 0.5g of the leaves crude extract was dissolved in 0.5ml dichloromethane to give a dilute solution and then 0.5ml of acetic anhydride added, followed by three drops of concentrated sulphuric acid. A blue-green coloration indicates the presence of steroids (MacNee, 2005).

3.9.7.2. Test for Tannins (Ferric chloride test)

A 0.5g of the leaves crude extract was dissolved and added to a tube containing 20ml of boiling distilled water and then boiled for an hour. A few drops of ferric chloride was added and allowed to stand for proper color development. A blue black coloration indicates the presence of tannins (Alebiosu and Yusuf, 2015).

3.9.7.3. Test for Alkaloids (Wagner's Test)

Filtrates were treated with Wagner's reagent (Iodine in Potassium Iodide). Formation of brown/reddish precipitate indicates the presence of alkaloids (Puri and Kumar, 2019).

3.9.7.4. Test for Saponins (Frothing test)

Saponins was tested by dissolving about 0.5g of the leaves crude extract in a test tube containing 3ml of hot distilled water and then the mixture was shaken vigorously for one minute and persistent foaming that indicates the presence of saponins (Solanki et al.,2019).

3.9.7.5. Test for Flavonoids (Cyanidine test)

One half gram (0.5g) of the leaves crude extract was dissolved in methanol and 2ml of concentrated hydrochloric acid added. A spatula full of magnesium turnings was added and the mixture observed for effervescence. A brick red coloration observed indicates the presence flavonoids (Christen, 2000).

3.10. Fractionations of Compound

3.10.1. Silica Gel Column Chromatography

The grounded powder of ethanol leaf extract of *A. salicifolia* (13.0g) was adsorbed on equal amount of silica gel (60-120 mesh size) and subjected to silica gel column chromatography (120g silica gel); silica gel (120g) was mixed with 200 mL of Dichloromethane to form a homogenous suspension/slurry and stirred using a glass-stirring rod to remove bubbles. The silica gel slurry was then poured into a glass column. Elution of the extract was done with increasing gradient of ethanol in Dichloromethane. Each fraction was collected and labeled and the purity of all fractions was analyzed with TLC. In TLC analysis, TLC Silica gel 60 was used as a solid phase and a mixture of the solvents hexane: ethyl acetate (v: v) was used as

mobile phase. Fraction that showed similar TLC profiles and the same characteristic color on TLC were combined and dried using rotary evaporator at temperature of about 40-45⁰C to get extraction of each solvent. Therefore, based on TLC profiles, Fraction 4 (hexane: EtOAc = 8:2, Rf value of 0.28) furnished compound **1** (N-acetyl chitobiose derivative **1**) (134mg), Dichloromethane: ethanol (40:60) as eluent.

3.10.2. Structure Elucidation of Isolated Compound

The structure of pure compound (was determined using ¹H-, ¹³C- and DEPT-135 spectra recorded on a Bruker advance 400 spectrometer operating at 400 MHz in CDCl₃ and DMSO-d₆. All chemical shifts were stated in parts per million (ppm) and coupling constants were expressed in Hz.

3.11. Data analysis

The mean and standard error of the mean (mean $\pm$ SEM) of the measurement was used to present the findings of the agar disk diffusion assay. The difference among mean values were assessed using one way analysis of variance (ANOVA) using SPSS version 20 (Statistical Package for Social Services, Chicago, IL, USA).

CHAPTER 4

RESULTS

4.1. Plant Extracts Yield

Acetone, ethanol and aqueous extraction gave different extraction yields. *A. salicifolia* leaf had the highest yield (7.5%), followed by roots of *P. reticulatus* (6.3%). The lowest extraction yield was obtained with *P. reticulatus* (3.8%) (Table 3).

Table 3. Yield of extraction (%) of different extracts of two plants.

Plants	Parts used	Yield of extraction (%)		
		Acetone	Ethanol	Aqueous
<i>A. salicifolia</i>	Leaves	7.5%	5.6%	5.3%
<i>P. reticulatus</i>	Roots	4.8%	6.3%	3.8 %

4.2. Antimicrobial Activities of *Agarista salicifolia* against Test Pathogens by Disk Diffusion Methods

The leaf extracts of *A. salicifolia* was tested for their antimicrobial properties against the test pathogens. As can be seen in Table 4, the diameters of the zones of inhibition ranged from 11-25.3 mm for ethanol and from 8.7-16.3 mm for acetone crude extracts of leave of *A. salicifolia*. Water based leave extracts had antimicrobial activity that ranged from 8.7-15.3 mm. Compared to positive controls (21-27.3 mm) for gentamicin and 25.7 mm for Ketoconazole. The negative control (DMSO) showed no inhibition against all the bacterial and fungal test pathogens. The acetone leave extract of *A. salicifolia* showed maximum zone of inhibition against *S. typhimurim* (16.3±1.0 mm) and minimum zone of inhibition against *C. albicans* (8.7±2.9 mm). Ethanol leave crude extracts of *A. salicifolia* showed maximum zone of inhibition against *S. aureus* (25.3±1.2 mm) and minimum zone of inhibition against *C. albicans* (11±1.52 mm). Water leave extracts of *A. salicifolia* showed maximum zone of inhibition against *S. pyogens* (15.3±1.5 mm) and minimum zone of inhibition against *S. aureus* (8.7±0.8 mm).

As indicated in Table 4, ethanol leave extracts of *A. salicifolia* leaves show that the zones of inhibition for *S. aureus* and *S. typhimurim* were significantly higher ($p<0.05$) than those observed against *S. pyogens* and *P. aeruginosa*. Similarly, the acetone leave extracts of *A. salicifolia* show that the zones of inhibition against *S. typhimurim* were significantly higher than ($p<0.05$) *S. pyogens*, *S. aureus* and *P. aeruginosa* at the same concentration (100mg/ml). Water leave extract of *A. salicifolia* shows less antibacterial activities against all bacterial pathogen except *S. pyogens*. *C. albicans* did show less antibacterial activities in acetone, ethanol and water leaves extracts of *A. salicifolia* at the same concentration (100mg/ml).

Table 4. Diameter of zones of inhibition (mm) of *Agarista salicifolia* leaves extracts against microorganisms at 100mg/ml concentration (mean $\pm$ SEM, n = 3).

Test pathogen	Zone of Inhibition (mm)					
	Disk diffusion method					
	AE 100mg/ml	EE 100mg/ml	WE 100mg/ml	GE 10mg/ml	KE 10mg/ml	DMSO 0.1ml
<i>S. aureus</i>	12 $\pm$ 1.16	25.3 $\pm$ 1.2	8.7 $\pm$ 0.8	26.3 $\pm$ 1.5	ND	0
<i>S. pyogens</i>	11.7 $\pm$ 1.49	13.7 $\pm$ 1.2	15.3 $\pm$ 1.5	27.3 $\pm$ 2.5	ND	0
<i>S. typhimurim</i>	16.3 $\pm$ 1.0	21.3 $\pm$ 0.88	10 $\pm$ 0.57	21.3 $\pm$ 1.5	ND	0
<i>P. aeruginosa</i>	11 $\pm$ 0.57	17.6 $\pm$ 0.88	9.7 $\pm$ 1.46	21 $\pm$ 0.57	ND	0
<i>C. albicans</i>	8.7 $\pm$ 2.9	11 $\pm$ 1.52	11 $\pm$ 0.28	ND	25.7 $\pm$ 1.2	0

Key: AE = Acetone leave extract, EE= Ethanol leave extract, WE = Water leave extract, GE = Gentamicin, KE = Ketoconazole, n = number of experimental replicates, SEM = standard error of the mean, ND = Not determined.

4.3. Antimicrobial Activities of *Phyllanthus reticulatus* against Test Pathogens by Disk Diffusion Methods

The antimicrobial activities of the ethanol, acetone and water crude extracts of the roots of *P. reticulatus* at concentrations of 100mg/ml were evaluated against the bacterial and fungal test pathogen. As indicated in Table 5, the zones of inhibitions of the ethanol, acetone and water root crude extracts of *P. reticulatus* were in the range of 10.7 – 17.66 mm, 8 – 12 mm and 5.33 – 9.3 mm, respectively. The antibiotics on the tested bacteria and fungus ranged from 15.6 – 31.6 mm and 21.3 mm for Gentamicin and Ketoconazole respectively.

Among the root crude extracts, the ethanol root extract of *P. reticulatus* showed maximum zone of inhibition against *S. aureus* (17.7±1.20 mm) and minimum zone of inhibition against *P. aeruginosa* (10.7±2.92 mm). The acetone root extract of *P. reticulatus* showed maximum zone of inhibition against *S. pyogens* (12±0.57 mm) and minimum zone of inhibition *S. typhimurim* (8±0.91 mm). Water root extracts of *P. reticulatus* showed maximum zone of inhibition against *C. albicans* (9.3±0.33 mm) and minimum zone of inhibition against *S. aureus* (5.33±0.33 mm).

Table 5 also shows that the acetone root extracts of *P. reticulatus* show that the zones of inhibition for *S. pyogens* higher than those observed against *S. aureus*, *S. typhimurim* and *P. aeruginosa*. Similarly, the ethanol root extracts of *P. reticulatus* show that the zones of inhibition against *S. aureus* and *S. pyogens* were significantly higher than ($p<0.05$) *S. typhimurim* and *P. aeruginosa* at the same concentration (100mg/ml). Water root extract of *P. reticulatus* did show less antibacterial activities against all bacterial pathogen. However, the ethanol root extracts of *P. reticulatus* show that the zones of inhibition against *C. albicans* were significantly higher than ($p<0.05$) acetone and water root extracts of the *P. reticulatus* at (100mg/ml).

Table 5. Diameter of zones of inhibition (mm) of *Phyllanthus reticulatus* root extracts against microorganisms at 100mg/ml concentration (mean $\pm$ SEM, n = 3).

Test pathogen	Zone of Inhibition (mm)					
	Disk diffusion method					
	AE	EE	WE	GE	KE	DMSO
	100mg/ml	100mg/ml	100mg/ml	10mg/ml	10mg/ml	0.1ml
<i>S. aureus</i>	8.7 $\pm$ 0.88	17.7 $\pm$ 1.20	5.33 $\pm$ 0.33	31.6 $\pm$ 1.35	ND	0
<i>S. pyogens</i>	12 $\pm$ 0.57	16.3 $\pm$ 0.88	8.67 $\pm$ 0.88	25.3 $\pm$ 0.88	ND	0
<i>S. typhimurim</i>	8 $\pm$ 0.91	11.5 $\pm$ 1.06	6.3 $\pm$ 1.90	30.7 $\pm$ 0.87	ND	0
<i>P. aeruginosa</i>	11 $\pm$ 1.15	10.7 $\pm$ 2.92	7 $\pm$ 0.58	15.6 $\pm$ 0.9	ND	0
<i>C. albicans</i>	10 $\pm$ 0.00	15 $\pm$ 0.58	9.3 $\pm$ 0.33	ND	21.33 $\pm$ 0.8	0

Key: AE = Acetone root extract, EE= Ethanol root extract, WE = Water root extract, GE = Gentamicin, KE = Ketoconazole, n = number of experimental replicates, SEM = standard error of the mean, ND = Not determined.

4.4. Minimum Inhibitory Concentration (MIC) of the Crude Extracts

The data shown that the MIC of ethanol, water and acetone crude extracts of plants against the tested bacterial and fungal pathogens with the interval of from 100 mg/ml to 12.5mg/ml (Figure 9). At 25 mg/ml, acetone leave extracts (against *S. aureus*) and ethanol leave extract of *A. salicifolia* (against *S. aureus* and *S. pyogen*) and ethanol root extract of *P. reticulatus* (against *S. aureus*) shared the least MIC. On the basis of preliminary findings (disk diffusion and MIC values), the ethanol leaves extract of *A. salicifolia* was further selected in order to determine the chemical constituents responsible for antimicrobial activity by using spectroscopic methods (NMR) and for the following bioassay analysis since, the relatively low MIC values recorded for the *A. salicifolia* leave extracts against the test pathogens verifying the high activity of the extract.

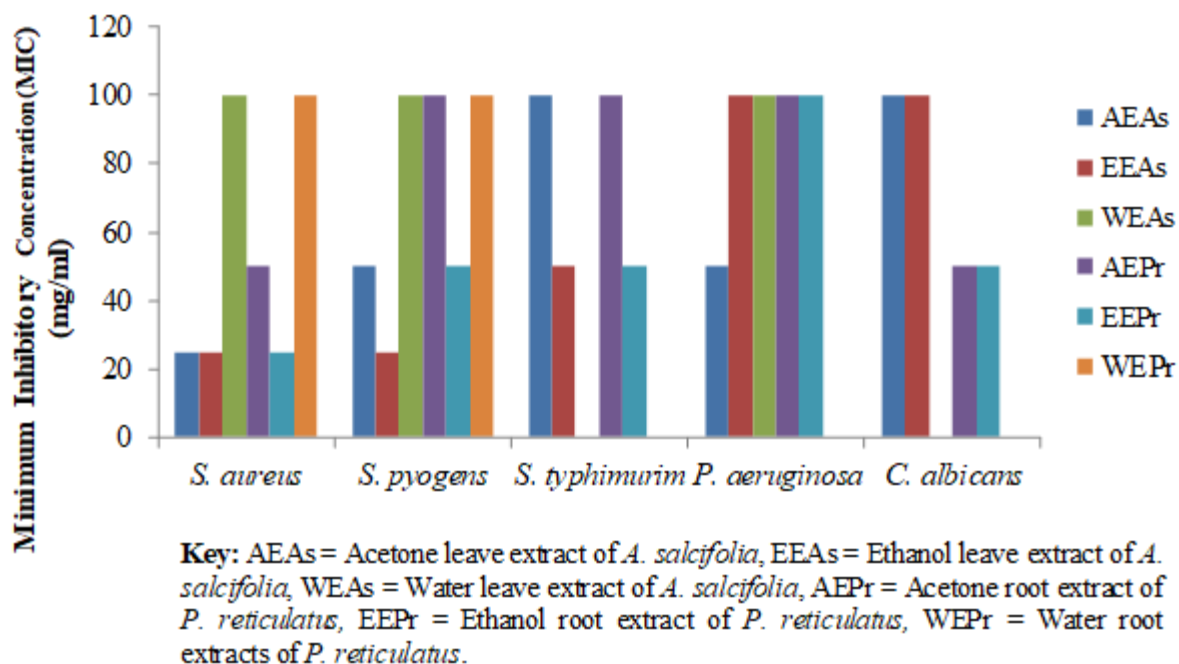


Figure 9. Minimum Inhibitory Concentration (MIC) of leaves extracts of *A. salicifolia* and root extracts of *P. reticulatus* against bacterial and fungal test pathogens.

All bacterial strains, *S. aureus*, *S. pyogens*, *P. aeruginosa* and *S. typhimurim* were found to be susceptible to both the ethanol leave extract and purified compound of *A. salicifolia* but *C. albicans* was resistant to both ethanol leave extract (C) and the purified compound of *A. salicifolia* by column (C1) at the same concentrations (5mg/ml) shown in (Figure 10).

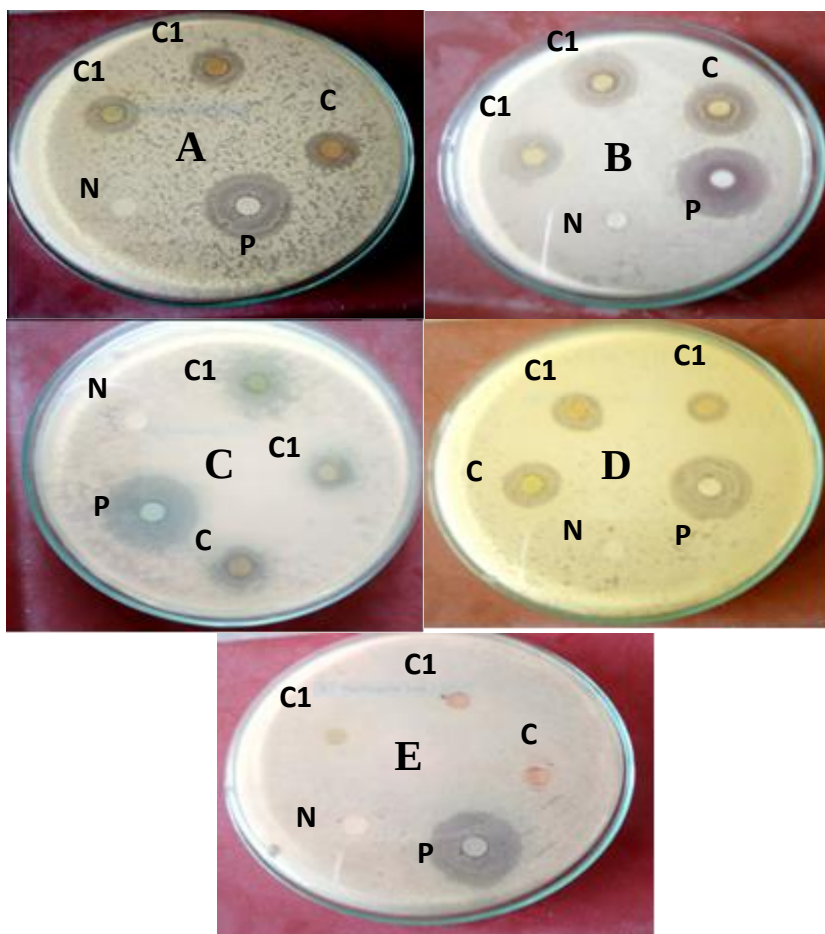


Figure 10. Diameter of inhibition zone (mm) of ethanol leave crude extract (C) and purified compound (C1) of *A. salicifolia* on *S. typhimurim* (A), *P. aeruginosa* (B), *S. aureus* (C), *S. pyogens* (D) and *C.albican* (E) respectively at 5mg/ml. (P) indicated positive control and (N) indicated negative control.

4.5. Anti-Biofilm Assay

4.5.1. Test Tube Method

The highest anti-biofilm activity was shown by 30 mg/ml *A. salicifolia* ethanol leave extract on *S. typhimurim* followed by *S. pyogen* but strong biofilm formation by *C. albicans* succeed by little formation by *P. aeruginosa* and *S. aureus* (Figure 11).

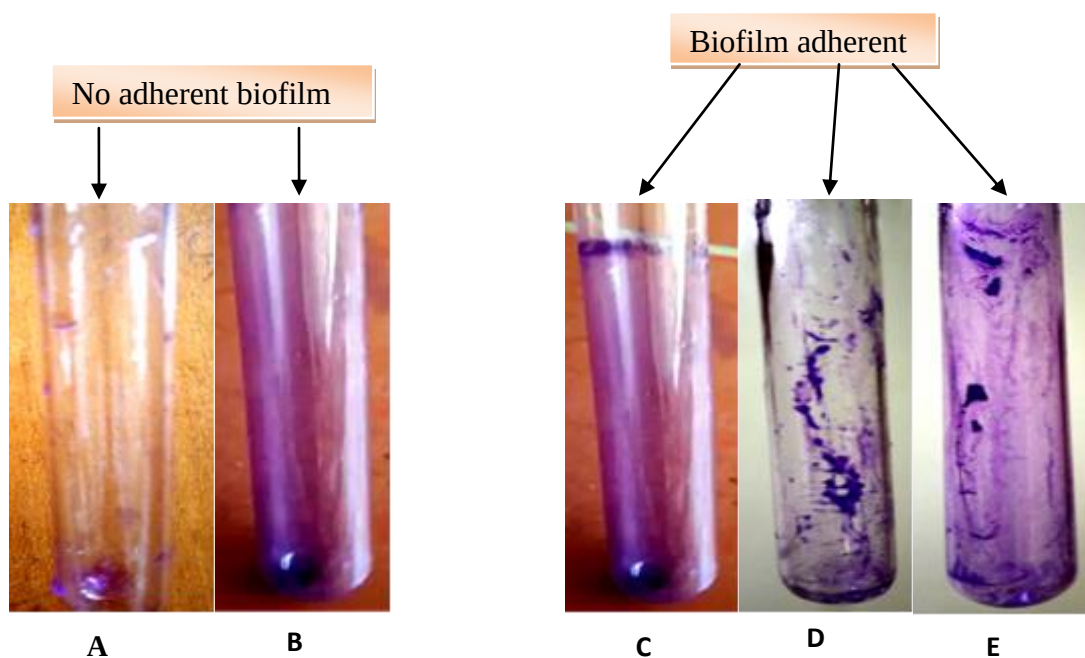


Figure 11. Anti-biofilm activities by *A. salicifolia* leave extracts against *S. typhimurim* (A), *S. pyogen* (B), *S. aureus* (C), *P. aeruginosa* (D) and *C. albicans* (E) through crystal violate assay.

4.5.2. Swarming Motility Assay

Figure 12 indicates that *A. salicifolia* leaves ethanol extract has major impact on the swarming motility. Highest anti-swarming activities was shown by 50mg/ml leave extract of *A. salicifolia* against *S. typhimurim*, *P. aeruginosa* and *C. albicans* but in the similar concentration (50mg/ml) *S. aureus* and *S. pyogens* showed less swarming inhibition activity.

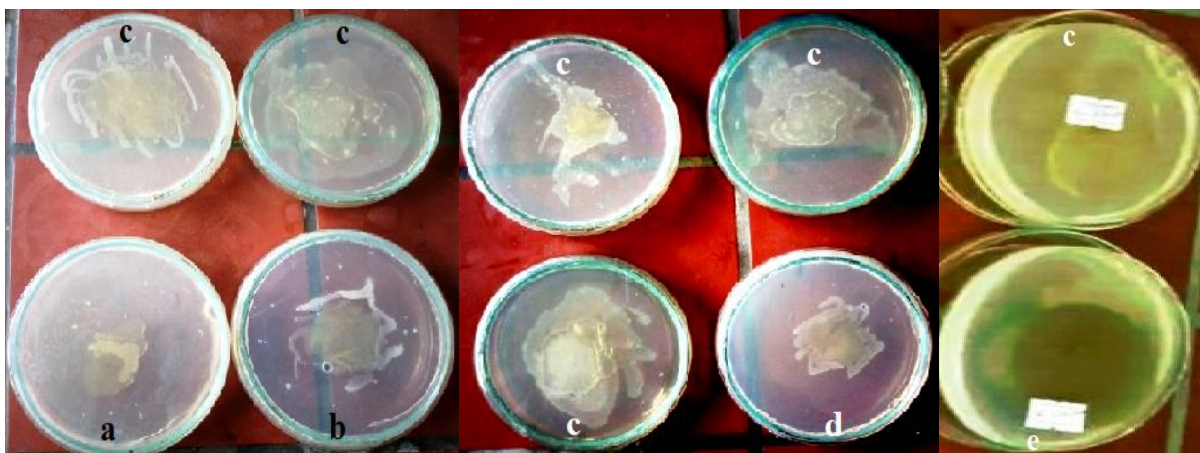


Figure 12. The movement of *S. aureus*, *S. pyogens*, *S. typhimurium*, *P. aeruginosa* and *C.albicans* was perceived though they were incubated for 24 h at the same concentrations (50mg/ml) of *A. salicifolia* leaves extract. Swarming motility of (A) *S. aureus*, (B) *P. aeruginosa*, (C) *S. pyogens*, (D) *S. typhimurium* (E) *C. albicans* in the presence of *A. salicifolia* leave extracts (left to right; control (C) : without extract top).

4.6. Bioassay for Anti-Quorum Sensing

4.6.1. Preliminary Screening (Disk diffusion assay)

Cloudy circle was shown by *S. aureus*, *S. pyogen* and *S. typhimurium* but *P. aeruginosa* did not show circle (Figure 13) around the leave extracts at the concentrations of 5mg/ml loaded disc. The negative control (LB broth loaded disc) did not show any inhibition of violacein production around the disk when the positive control showed very clear, visible inhibition.

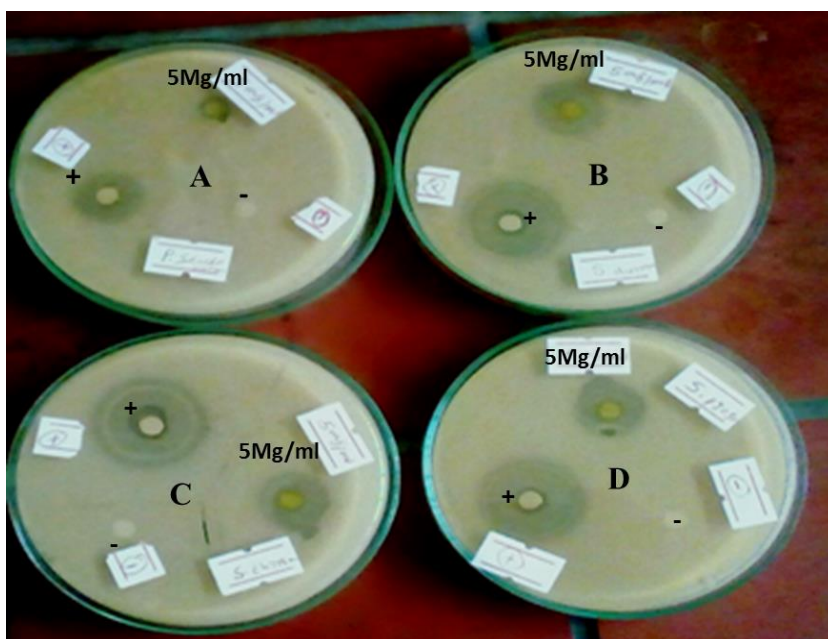


Figure 13. Anti-quorum sensing activity by *A. salicifolia* leaves extract against *S. aureus* (B), *S. pyogen* (D), *S. typhimurium* (C) and *P. aeruginosa* (A) at the concentrations of 5mg/ml using agar disk diffusion method. (+) indicated positive control and (-) indicated negative control.

4.7. Qualitative Antibacterial Activity Assay by Bioautography

The hexane and ethyl acetate solvent system separated the faded active bands against *P. aeruginosa* better than the *S. aureus* (Figure 14).

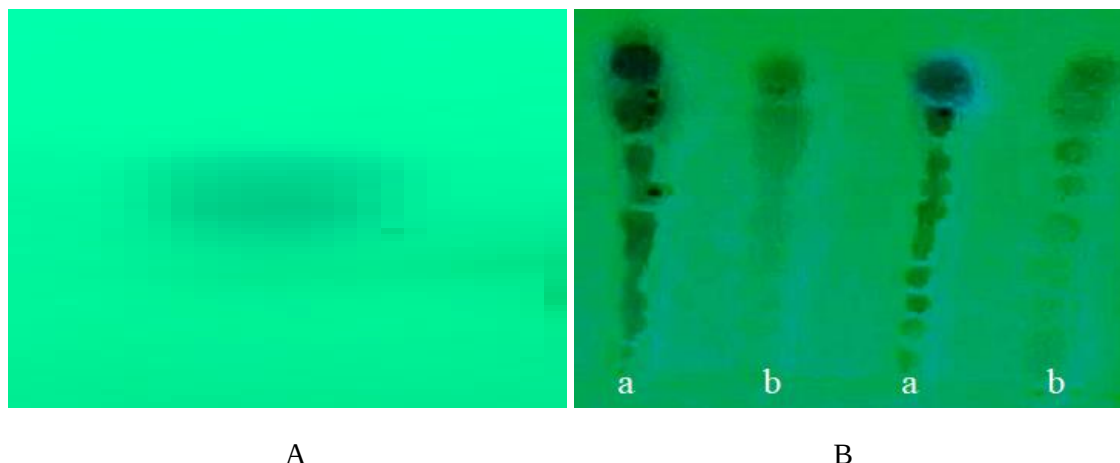


Figure 14. (A) Chromatogram developed in hexane: EtOAc (8:2) solvent system of the *A. salicifolia* leaf ethanol extracts. (B) Bioautography of *S. aureus* (A) and *P. aeruginosa* (B) developed with hexane: EtOAc (8:2); bleached bands show compound that inhibit the growth of the bacteria.

4.8. Phytochemical Screening

The results revealed that steroids, tannins, saponins and flavonoids were present in the ethanol leaves extracts of *A. salicifolia* (Table 6).

Table 6. Phytochemical tests results

Class of secondary metabolites	Test method used	Ethanol leaves extracts of <i>A. salicifolia</i>
Saponins	Frothing test	+
Flavonoids	Cyanidine test	+
Steroids	Lieberman-Burchard test	+
Tannins	Ferric chloride test	+
Alkaloids	Wagner's Test	-

(+) indicated presence and (-) indicated absence

4.9. The Nuclear Magnetic Resonance (NMR) Spectrum Analysis of Pure Compound Isolated from Leaves of *A. salicifolia*

4.9.1. Characterization of Bioactive Compound

The compound was isolated as yellowish solid with melting point of 184-186°C. The R_f value of 0.28 (Dichloromethane: ethanol (40:60), as eluent). The ¹H-NMR spectrum displayed (Appendix 1). Four anomeric proton signals at δ 5.19, 4.95, 4.59 and 4.57. The signal at 5.19 ppm ($J=3.8$ Hz) indicated that sugar residue had α -glycosidic configuration, and other residues also had α -glycosidic configurations according to their coupling constants ($J=3.4$ Hz). The ¹³C-NMR spectrum (Appendix 2) showed 19 carbon signals, including three anomeric carbons at δ 98.09 (C-1), 96.45, and 92.43. One carbonyl carbon was observed at δ 178.62 which didn't appear in DEPT-135 spectra which suggests that the compound have one carbonyl carbon, Thus, on the basis of signals observed at δ 5.19 and 178.62 it is suggested that N-Ac group is present (methyl group and carbonyl group, respectively).

This is also in good agreement that C-2 resonances shifted to δ 73.43 due to the influence of the attached amino group at C-2 position. The chemical shift value of C-4 signals shifted to δ 74.68 due to glycosylation at position 4. Similarly, another peak at δ 76.10 suggest there is second glycosylation at C-4' position suggesting the compound is N-acyltrisacharide (in agreement with observation of three major anomeric carbons signals). Three oxygenated methylenes were observed at δ 61.20, 67.83 and 68.56 were observed in DEPT-135 spectrum pointing down wards (Appendix 3). Thus, based on the above spectra data and comparison with literature the compound was identified to be identical with N-acetyl chitobiose derivative where only the methyl at C-5" position is oxidized to oxymethylene (-CH₂-OH), supported by DEPT-135 spectra (Appendix 3) (Figure 15).

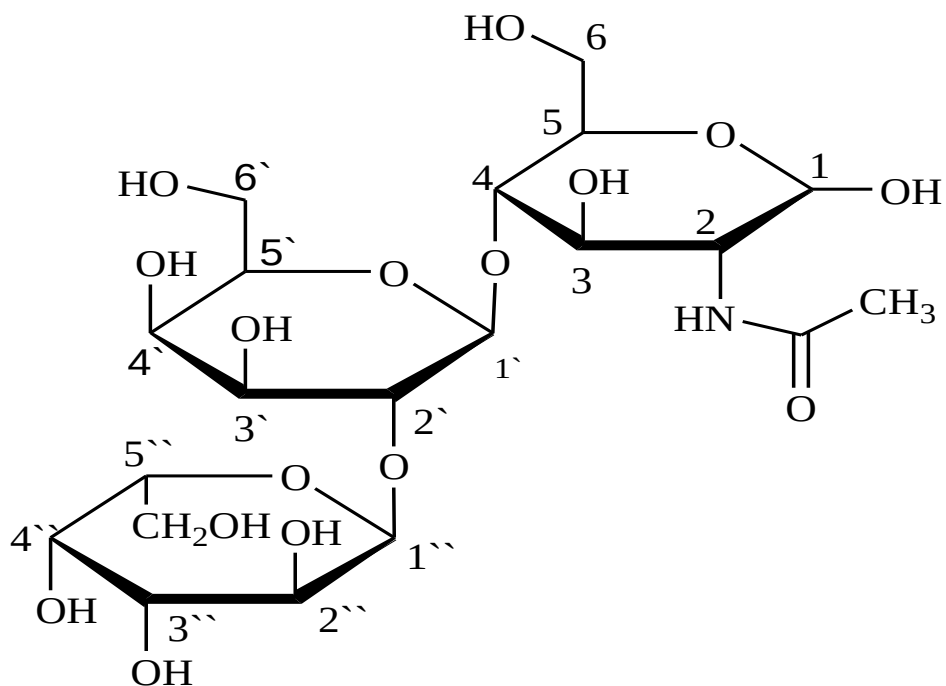


Figure 15. Chemical structure of N-acetyl chitobiose derivative (1).

Table 7. ^1H and ^{13}C NMR chemical shift values of compound (1) in CD_3OD .

Carbon position	Compound 1		N, N-diacetyl chitobiose (Saito et al., 1981)	
	δH	δC	δH	δC
C-1	(δ 5.19)	(δ 98.09)	(δ 5.31)	(δ 95.0)
C-2	(δ 3.38)	(δ 73.43)	(δ 3.46)	(δ 56.3)
C-3	(δ 3.45)	(δ 72.02)	(δ 3.43)	(δ 72.6)
C-4	(δ 3.70)	(δ 74.68)	(δ 3.94)	(δ 79.7)
C-5	(δ 4.59)	(δ 76.10)	(δ 4.10)	(δ 74.7)
C-6	(δ 3.23/3.21)	(δ 73.54)	(δ 3.93)	(δ 60.2)
C-1'	(δ 4.95)	(δ 102.59)	(δ 5.19)	(δ 101.6)
C-2'	(δ 3.43)	(δ 71.2)	(δ 3.33)	(δ 55.8)
C-3'	(δ 3.94),	(δ 68.7)	(δ 3.64)	(δ 73.6)
C-4'	(δ 4.10),	(δ 73.9)	(δ 3.82)	(δ 69.9)
C-5'	(δ 3.93/3.75)	(δ 67.83)	(δ 3.74)	(δ 76.0)
C-6'	(δ 3.93/3.75)	(δ 65.79)	(δ 3.86)	(δ 60.8)
C-1''	(δ 3.64)	(δ 78.7)	(δ 3.54)	(δ 93.0)
C-2''	(δ 3.72),	(δ 72.5)	(δ 3.78)	(δ 60.3)
C-3''	(δ 4.1),	(δ 68.8)	(δ 3.90)	(δ 68.8)
C-4''	(δ 3.83)	(δ 71.2)	(δ 3.68)	(δ 77.0)
C-5''	(δ 3.73)	(δ 61.20)	(δ 3.74)	(δ 71.7)
C-6''	(δ 3.34)	(δ 71.73)	(δ 3.59)	(δ 59.4)

4.10. DISCUSSION

The study revealed that ethanol, acetone and water leaves extract of *A. salicifolia* inhibited the growth of the test microbes. However, their inhibition potential and pattern is not the same which could be due to the difference in bacterial strain and the type of solvent used in plant part extraction (Manilal et al., 2016). Compounds extracted during the extraction procedure are mainly dependent on the type of the solvent. Water is the primary solvent used in the traditional medicine, but in the present study, compounds extracted in organic solvents (ethanol and acetone) have exhibited more significant antimicrobial activity compared to those extracted in water. In this study, ethanol extract of *A. salicifolia* leaves showed maximum inhibition zone against *S. aureus* (25.3 ± 1.2 mm). This finding was in agreement with the study of Getahun and Masresha, 2010 who reported ethanol and acetone extract of *A. salicifolia* leave showed maximum inhibition zone against *S. aureus*. The antibacterial activity of water leaves extracts of *A. salicifolia* against *S. pyogens* (15.3 ± 1.5 mm) and *S. aureus* (8.7 ± 0.8 mm) was also coincides with the previous study of Kang et al., 2011, reported that water extracts of *A. salicifolia* leaves was found to have antibacterial activity against gram positive bacterial species.

The crude extract and purified compound (N-acetyl chitobiose derivative) of leaves of *A. salicifolia* showed antibacterial activities against all test bacterial spp. i.e., *S. typhimurium*, *P. aeruginosa*, *S. aureus* and *S. pyogen*. However, both the crude extract and the purified compound showed that insignificant or no inhibition against *C. albicans* implying *C. albicans* would be resistant to the crude extracts and the purified compounds. The data generally suggested that *C. albicana* was more resistant than all bacteria to the crude extracts obtained from the leaves of *A. salicifolia*. The difference in the antibacterial and antifungal activities of the extracts could be due to the difference in the nature of the microorganism or the effectiveness of extracting solvent (Alzoreky and Nakahara, 2003).

Crude extracts obtained using ethanol and acetone root extracts of *P. reticulatus* showed higher inhibition zone than water crude extracts. This could be due to the higher volatility nature of ethanol and acetone which have higher tendency to extract more active compounds from the plant parts than water (Ibekwe et al., 2000). The ethanol root extract of *P. reticulatus* showed zone of inhibition against *S. aureus*. A study conducted by Sankannavar et al., 2012,

also showed that the ethanol extract of *P. reticulatus* root had maximum zone of inhibition, a finding which was in agreement with the present study. This may be due to ability of ethanol to extract bioactive compounds like tannis, saponnins, flavonoid and steroid which have higher concentrations in roots of *P. reticulatus* (Sheng et al., 2010). In the study of Shruthi et al., 2010, the ethanol root extract showed inhibition against *S. aureus* and *S. typhimurium*. Additionally, the finding of this study coincides with the study of several investigators (Sheng et al., 2010; Aarthi and Ramesh, 2017) who have reported acetone extract of the root of *P. reticulatus* inhibited the growth of *S. pyogen*. Inhibition of *C.albicans* by water extract of root of *P. reticulatus* was also reported by few previous investigators which agreed with the finding of present study.

Generally, the plant extracts obtained using ethanol and acetone exhibited better results than water extracts. This could be due to their capability to efficiently degrade the cell wall which has non-polar character. Furthermore, alcohols were found to be easier to pass the cellular membrane to extract the intracellular contents from the plant material and the presence of non-polar residues in the extracts, which had higher antibacterial activities. The present results also showed that different plant assayed possess different levels of antimicrobial activities. Among the extracts of the two plants, the leave extracts of *A. salicifolia* showed higher range of antibacterial activities than the root extracts of *P. reticulatus*. When comparing tested bacteria, *S. aureus* and *S. pyogen* was highly susceptible bacterial strain, while *S. typhimurim* and *P. aeruginosa* were less sensitive. Relatively, *C. albicans* was resistant fungal pathogens for all crude extract of *A. salicifolia* and *P. reticulatus* plants.

The likely reason for the difference in the activity of plant extracts could be the difference in type of plant species or bioactive compounds which could not be found uniformly among different plants, effectiveness of extracting solvent and nature of the microorganism (the difference in the sensitivity between Gram-negative and Gram-positive bacteria may be due to the variation in their cell wall structure). The Gram-positive bacterial cell wall comprises of 70–100 layers of peptidoglycans. Peptidoglycan is consisting of two polysaccharides, N-acetyl-glucosamine and N-acetyl-muramic acid cross-linked by peptide side chains and cross bridges which more hydrophilic and aide polar compounds enter. On the other hand, Gram-negative bacteria have a cell wall composed of a thin layer of peptidoglycan surrounded by an

outer membrane, highly hydrophobic. This outer membrane of gram-negative bacteria contains a unique component, lipopolysaccharide which serves as defense against many antibiotics. Furthermore, gram negative bacterial resistance may also originates from the secretion of the lactamase enzyme in the periplasmic space between the thin outer membrane and the cytoplasmic membrane (Henley et al., 2014).

The relatively low MIC values (25 mg/ml) recorded for the *A. salicifolia* leave extracts against the test pathogens verify the high activity of the extract at low concentrations. In one study, extracts of leaves of *A. salicifolia* were active at low MIC against both gram positive and negative bacteria have been reported by Kang et al. (2011). This activity may be attributed to the rich plant contents of active components such as tannins, saponins, alkaloids and flavone. Extracts with lower MIC scores are very effective antimicrobial agents. The higher MIC observed for *S. typhimurium*, *P. aeruginosa* and *C. albicans* in this study could be due to increased resistance to some of the bioactive ingredients in the plant. MIC is significant because populations of bacteria and fungus exposed to an insufficient concentration of the extract can develop resistance to antimicrobial agents. The high activity of antimicrobial agents at low concentrations is very critical in search of new drugs for chemotherapeutic as their toxicity is thought to be low to patients administered with such agents.

In the present study for biofilm showed that the highest anti-biofilm activity was detected by ethanol leave extract of *A. salicifolia* against *S. typhimurim* followed by *S. pyogen*, however, strong biofilm formation was seen by *C. albicans* following by little formation by *P. aeruginosa* and *S. aureus*. The *A. salicifolia* leave extract inhibits biofilm formations by instigating either cell to cell attachment or plays roles in virulence of pathogens (Wu et al., 2014). This might due to the effect of phytochemicals of *A. salicifolia*. *A. salicifolia* exhibited significant potential as anti-biofilm agent, as it was active upon some gram-negative and gram-positive bacteria strains (*S. typhimurim* and *S. pyogen*). Studies demonstrated that there is biological rational between biofilm and swarming motility which work on a coordinate manner. Swarming motility has been characterized as a remarkable feature for the formation of biofilm which is flagella-dependent movement of bacteria and is important for cystic fibrosis acute infections, common cause of severe nosocomial infections. In this study, *A. salicifolia* extract reduced the swarming motility zone of *S. typhimurim*, *P. aeruginosa* and *C.*

albicans. It might be because of phytochemicals of *A. salicifolia* on flagella-related processes, namely, flagella biosynthesis, rotation, rafting and chemotaxis, etc. (Tremblay and Deziel, 2008) reported that swarming motility assays of *P. aeruginosa* are influenced by incubation temperature, % agar, pH, and drying time under laminar flow.

This study showed that the leaves of *A. salicifolia* extract inhibited the production of violacein in agar diffusion assay. Cloudy circle was observed in *S. aureus*, *S. pyogen* and *S. typhimurium* compared with untreated at the concentrations of 5mg/ml using agar disk diffusion method (Figure 13). No much work has been done on anti-biofilm and anti-QS activity of the leaves extract of *A. salicifolia*. To my knowledge this study would be the first; no reports are available regarding the anti-biofilm activity of *S. aureus* *P. aeruginosa* and *C. albicans* by *A. salicifolia* leaves extract. The anti-QS potential of plant extracts to discover the potential of possible use in monitoring harmful pathogenic bacteria such as: *S. aureus*, *S. typhimurim* and *S. pyogen* etc. The extracts which inhibited biofilm formation and Swarming motility by pathogenic bacteria tested in this study was used for determining the inhibition of quorum sensing as an alternative strategy for controlling bacterial virulence as suggested by Vasavi et al. (2014). Therefore, this anti-biofilm inhibition and anti-QS evidences can be an alternative to combat infectious disease and it can be expected more with its amazing functions. The polar solvent system separated the active bands against *P. aeruginosa* better than the *S. aureus* (Figure 14), indicating that the bioactive compound are relatively polar. The retention factor (Rf), was calculated by dividing the distance moved by the compound by the distance of the solvent front. This represents the position of antimicrobial constituents on the chromatogram (Adamu et al., 2014). The number of active bands against *P. aeruginosa* bioautography was present in the leaves extracts of *A. salicifolia* (Rf = 0.28).

Phytochemical studies have shown that the leaves extract of *A. salicifolia* contain bioactive constituents such as steroids, tannins, flavonoids and saponins thus, the maximum potency against the test pathogens. This is evident from the strong positive correlation between total phenolic content and bacterial growth inhibition zone. In agreement with study, (Kotzekidou et al., 2008) reported that *A. salicifolia* leaves extract contains flavonoids, phenols, tannins, saponins and terpenoids, each bioactive constituent exert antibacterial activity through different mechanisms.

Earliest research indicates that flavonoids may modify allergens, viruses, and carcinogens and so may be biological “response modifiers. *In vitro* studies show that flavonoids also have anti-allergic, anti-inflammatory, antimicrobial, anticancer, and anti-diarrheal activities (Spencer, 2008). Saponins seem to stimulate the immune system (Zhao et al., 1996). Tannins exhibit antioxidant, antimicrobial and antiviral effects (Sayyah and Hadidi, 2004). The plant extracts were also revealed to contain steroids, which are known to produce an inhibitory effect on inflammation (Savithramma and Linga, 2011). Therefore, presence of steroids, tannins, saponins and flavonoids may contribute to the antimicrobial activity of the ethanol crude extract of *A. salicifolia* plants.

Antimicrobial property demonstrated by the ethanol leave extract of *A. salicifolia* against different bacterial strains and fungi could be due to the presence of N-acetyl chitobiose derivative compounds and other bioactive ingredients such as steroids, tannins, flavonoids and saponins. N-acetyl chitobiose derivative compound are belong to various chemical categories in plant materials and work either individually or in combination to produce an antimicrobial activity (Ghosh et al., 2011). Another studies also showed that *A. salicifolia* contains potent antibacterial secondary metabolites (Moteriya et al., 2014). Therefore, the antimicrobial activity of the leaves extracts of *A. salicifolia* plants may be described by the total secondary metabolites contents. Many research reports reveal that secondary metabolites of plant extracts contribute to the antimicrobial properties whereby the degree of inhibition depends on the concentration of these compounds (Taguri et al., 2006).

The biological activities of N-acetyl chitobiose derivative compounds are related to the presence of hydroxyl groups (-OH) and phenolic ring in the molecular structure. Membrane proteins of bacteria interact with hydroxyl groups of phenolics by hydrogen bonding. This interaction alters the membrane permeability that in turn causes loss of cellular constituents and cell destruction (Marcucci et al., 2001). From this it can be assumed that phenolic compounds act at two different points (cell membrane and cell wall), affecting the growth and metabolism of bacteria (Stefanovic et al., 2012). Similar results have also been reported in other studies (Rajeshwar et al., 2005).

CHAPTER 5

CONCLUSIONS AND RECOMMENDATION

5.1. CONCLUSIONS

The findings of this study revealed that the leaves crude extracts of *A. salicifolia* and root extracts of *P. reticulatus* exhibited significant antimicrobial activity against the four bacterial pathogen (*S. aureus*, *S. pyogens*, *S. typhimurium* and *P. aeruginosa*) and one fungal pathogen (*C. albicans*). The three solvents employed for the extraction process i.e., acetone, ethanol and water have showed different power in their extraction efficiency which could be due to their difference in polarity. The highest antimicrobial activity was observed for ethanol leave extract of *A. salicifolia*. The ethanol leave extracts of *A. salicifolia* also displayed anti-biofilm and anti-quorum sensing activity against the test pathogen. The antimicrobial property shown by the ethanol leave extract of *A. salicifolia* against different pathogens could be due to chemical entity of N-acetyl chitobiose derivative with a broad spectrum of antimicrobial activity. It can be concluded that the existence of N-acetyl chitobiose derivative compound and other bioactive constituents in the *A. salicifolia* leave extract may be responsible for their significant antimicrobial action. Generally, the result of the present study confirmed the traditional use of these plants against various microbial infections by the people therefore; *A. salicifolia* is a better source of antimicrobial agent hence can be of interest in the development of new chemotherapeutic drug.

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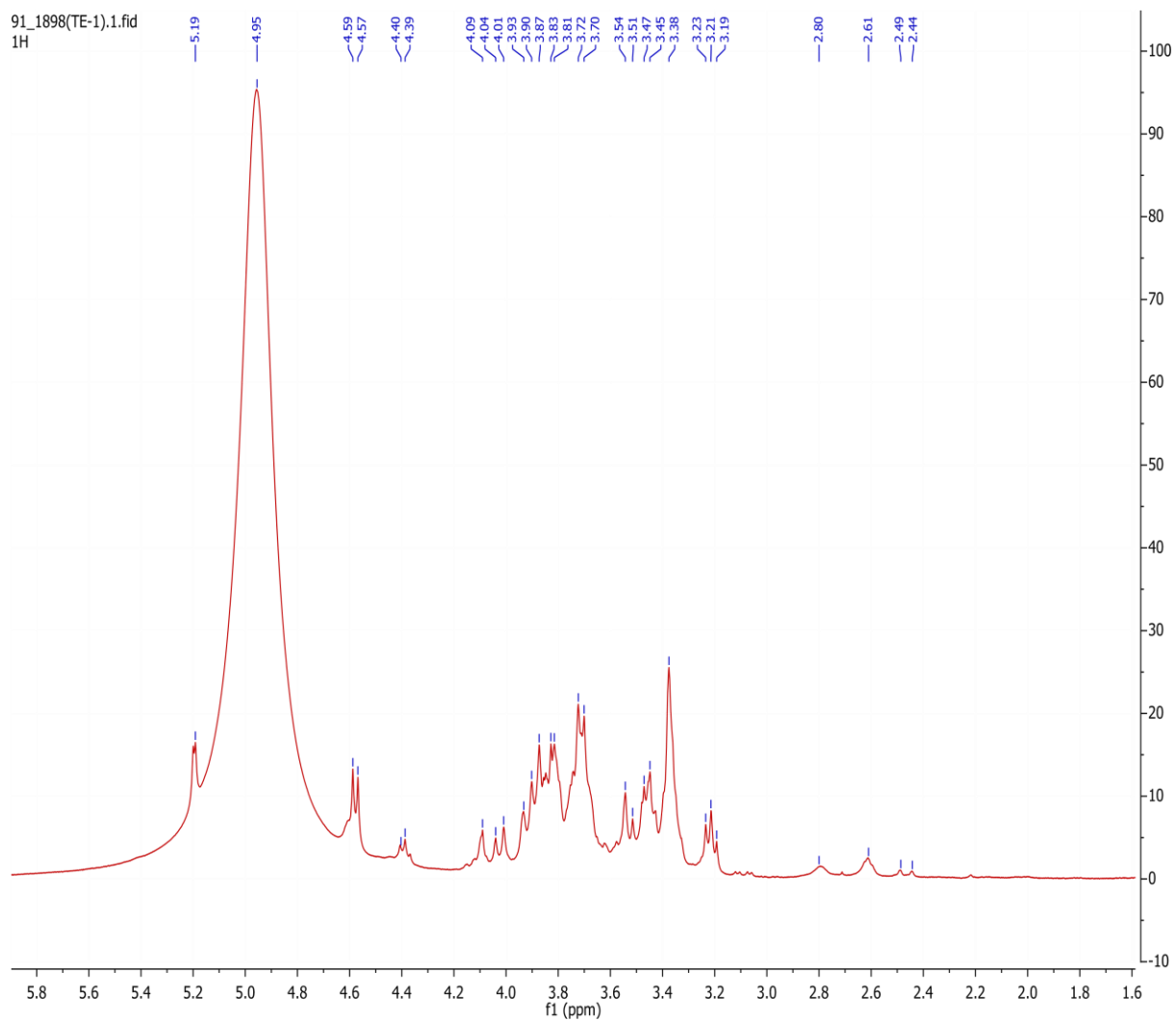
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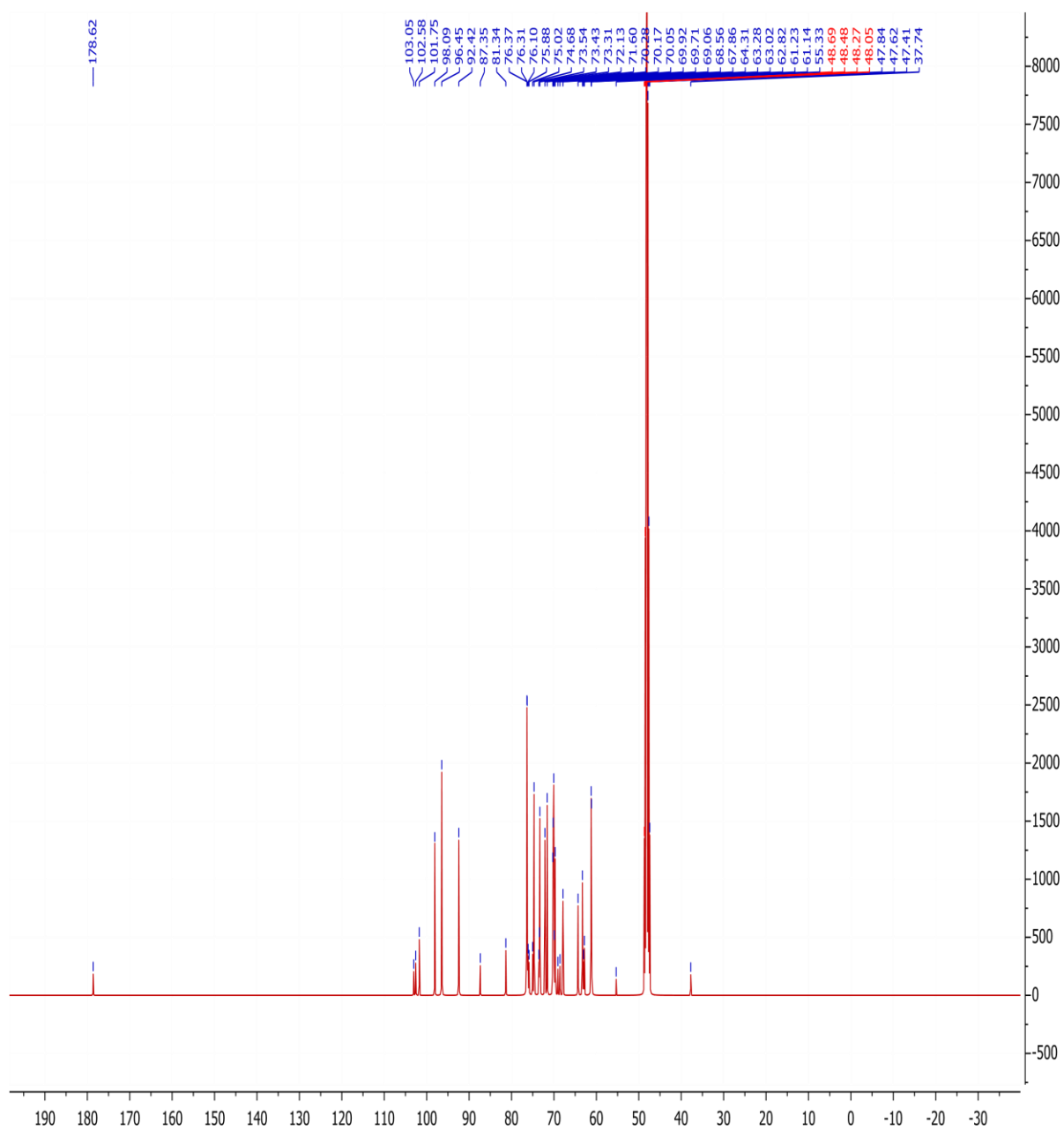
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7. APPENDIXES

Appendix 1: ^1H -NMR spectrum of compound



Appendix 2: ¹³C-NMR spectrum of compound



Appendix 3: DEPT-135 spectrum of compound

