Isolation of Bioactive Compounds from *Ekebergia capensis*, *Catha edulis* and *Premna resinos*a for Antimicrobials activity Test

By: Abu Feyisa Meka

A Thesis Submitted to the Department of Applied Biology

School of Applied Natural Science

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

Office of Graduate Studies

Adama Science and Technology University

September 2018

Adama, Ethiopia

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Candidate Declaration

I hereby declare that the work which is being presented in this research entitled as **Isolation of Bioactive Compounds from *Ekebergia capensis, Catha edulis* and *Premna resinos*a for Antimicrobials activity Test** in partial fulfillment of the requirement for the award of the degree of MSc in Microbiology. It was authentic record of my original work carried out from September 2017 to July 2018 under the advisors of Dr. Teshome Geramow, Department of Applied Biology and Dr. Yadessa Melaku, Department of Applied Chemistry, School of Applied Natural Science, Adama Science and Technology University. The matter embodied in this has not been submitted by other person or me for the award of any other degree. All relevant resources of information used in this body have been duly acknowledged.

_____	_____	_____
Candidate	Signature	Date

ADAMA SCIENCE AND TECHNOLOGY UNIVERSITY

SCHOOL OF APPLIED NATURAL SCIENCES

APPLIED BIOLOGY DEPARTMENT

We, the undersigned, members of the Board of Examiners of the final open defence by Abu Feyisa have read and evaluated his thesis entitled "**Isolation of Bioactive Compounds from *Ekebergia capensis*, *Catha edulis* and *Premna resinos*a for Antimicrobials activity Test**" and examined the candidate. This is, therefore, to certify that the thesis has been accepted in partial fulfilment of the requirement of the Degree of MSc in Microbiology.

Advisor	Signature	Date
Co-advisor	Signature	Date
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Chairperson	Signature	Date
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List of Acronyms and Abbreviations

^{13}C-NMR	Carbon-13 Nuclear Magnetic Resonance
^{1}H NMR	Proton Nuclear Magnetic Resonance
AHL	Acyl-Homoserine Lactone
ANOVA	Analysis of variance
CC	Column Chromatography
CFU	Colony Forming Unity
CLSI	Clinical and Laboratory Standards Institute
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO	Dimethyl sulfoxide
LB	Luria Bertani
LD_{50}	Lethal $Dose_{50}$
LED	Light Emitting Diode
MHA	Mueller Hinton Agar
MIC	Minimum inhibitory concentration
NA	Nutrient Agar
NMR	Nuclear Magnetic Resonance
ppm	parts per million
QS	Quorum Sensing
SDA	Sabouraud Dextrose Agar
SDB	Sabouraud Dextrose Broth
TLC	Thin Layer Chromatography.

Abstract

Biofilm is the association of microbial cells that attach on extracellular polymeric substance matrix. Using complex mechanisms produced by specialized genes, biofilm members can communicate, exchange experience and virulence factors with each other a phenomenon which called quorum sensing. The main objective of this study was to Isolate bioactive compounds from locally available medicinal plants for their, anti-biofilm, and anti-quorum sensing activities. E. capensis, C.edulis and P. resinosa were collected from Oromia region North Shewa Kuyu woreda. The collected samples were dried and made powder. The powder was extracted using hexane, ethyl acetate and methanol for 72h at room temperature. All extracts were tested for antimicrobials, anti-biofilm and anti-quorum sensing activities. Antibiofilm were tested by test tube and swarming motility test methods and anti-quorum sensing were tested by disk diffusion and flask incubation test for violence inhibition production methods. QS inhibitory effect of extract on milk spoilage by Pseudomonas species and toxicity studies of extracts on leech were carried out. The overall yield of E. capensis, P. resinosa and C. edulis ethyl acetate extract were 1.72%, 0.90%, and 1.47%. The results indicate ethyl acetate extract of E. capensis and its three purified compounds exhibited strongest antimicrobials, antibiofilm and anti-quorum sensing activity. The antimicrobials activity of extract against S. aureus, C. albicans zone of inhibition ranged between 4-20mm. Strong biofilm formation by C. albicans and P. aeruginosa, when 10mg/ml of extract inhibited biofilm formation by S. aureus. Anti-swarming activities were shown by 10mg/ml of extract on P. aeruginosa, S. aureus and 10mg/ml extract showed highest inhibition of violacein production, 72. 6% on S. aureus and, when 67.74%, 69.35%, were shown by P. aeruginosa, C. albicans, respectively. Similarly, extract of E. capensis prevent spoilage of milk by Pseudomonas species. In addition, the extracts toxicity on leech show positive results. Taken together, this finding suggests ethyl acetate extract and its purified compounds from bark of E. capensis exhibit strong antimicrobials, anti-biofilm, anti-quorum sensing activity and prevent spoilage of milk value to food product. This was useful to manage infection as diseases caused by biofilm forming microorganisms.

Keywords: antimicrobials, biofilm, extraction, Plants, resistance

1. INTRODUCTION

1.1. Background of the Study

The discoveries of new antimicrobial agents are providing effective control of large number of lives treating infection diseases (Ling *et al*., 2015). Even though, recently multi drug resistance bacteria have been increased because of microbial resistance, antibiotic treatments became more challenging to produce and clinically implement. In recent research, the documented antibiotic resistance needs for the development of different target process in the pathogen. Such target systems are quorum sensing and biofilm formation (Tanver *et al*., 2017). Quorum sensing consists of cell-to cell communication that depends on the production and response to small diffusible molecule called auto-inducers. This can lead to the formation of biofilm and causing other virulence factors which are problems for food safety and biofilm related infection diseases (Tanver *et al*., 2017).

Considering the impact of quorum sensing and biofilm, several strategies (cleaning agent, phages, irradiation, essentials oil, etc.) have been developed in food factory and drug discovery (Tanver *et al*., 2017). To prevent spoilage of food and bacterial infection by using medicinal plants are safe and non-toxic regarding to health status. Further, blocking of quorum sensing signal transduction can be achieved by using an antagonist molecule capable of competing or interfering with the native Acyl-Homoserine Lactone (AHL) signal for binding to the LuxR (autoinducer-responsive transcriptional activator) type receptor (Koh *et al*., 2013).

Competitive inhibitors are structurally like the native AHL signal and can bind to and occupy the AHL-binding site but will fail to activate the LuxR-type receptor. The noncompetitive inhibitors show little or no structural similarity to AHL signals and these molecules bind to different sites on the receptor protein (Koh *et al*., 2013). Thus, quorum sensing inhibitors that can either target synthesis of the cell signaling molecules or block these signaling systems can be used to prevent spoilage of food and biofilm formation by food-related bacteria (Tanver *et al*., 2017)

The use of natural products as therapy against infectious diseases is a traditional therapeutic measure especially in developing countries as they contain a combination of potential antimicrobial compounds instead of a single purified molecule (Packiavathy *et al*., 2012). Thus, it has become inevitable to search for new and safer anti-QS compounds from natural products. Quorum sensing inhibitors have been reported in various natural products and medicinal plant species, in fruits and spices and phytochemicals (Packiavathy *et al*., 2012).

Although the food industry primarily uses essential oils as flavorings, they represent an interesting source of natural antimicrobials for food preservation. Plant derived essential oils are well-known antimicrobial agents. Their application as food preservatives to control food spoilage and food borne pathogenic bacteria is due to their growth inhibition property (Tanver *et al*., 2017). However, there are very few studies focusing on the development of novel intervention and food preservation techniques based on the anti-quorum sensing property of plant essential oils. Therefore, medicinal plants are popular for their new bioactive compound and therapeutic values. The antimicrobial activities of some of the tested crude extract and their constituents have been investigated previously (Tanver *et al*., 2017).

In Ethiopia, local people were used different medicinal plants to preserve food, treat infection diseases, and use as source of foods traditionally. These plants were collected from wild habitats and prepare either in dry form or fresh form remedies. Most of remedy preparations did not have additive substances while the remaining has different additive substances like honey, sugar, betters and oil for the treatment of single ailment (Information gathered from traditionally healer peoples or folk medicine). Based on traditional value of medicinal plants, this study was to isolate bioactive compounds from leaves of *Catha edulis*, root of *Premna resinosa* and bark of *Ekebergia capensis* for anti-biofilm, anti-quorum sensing and antimicrobial properties in which scientific studies on their and anti-biofilm and anti-quorum sensing activities are limited. Further, the inhibitory effects of crude extract of these three medicinal plants on biofilm were studied on milk spoiling *Pseudomonas* species and lethality and behavioral changes on leeches by crude extracts were also studied

1.2. Statement of the Problem

Antibiotic resistance has become a great problem due to an increasing incidence of multiple resistances in human pathogenic microorganisms in recent years, largely due to indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases (Gray *et al*., 2013). In addition to this, spoilage of foods and biofilm formation by food spoilers and pathogens are major problems in the food processing industry (Koh *et al*., 2013). Drugs from plants sources are key way towards addressing this problem.

Different medicinal plants were used in Ethiopia by local people at different places for different purpose. Because of bioactive compounds that are present in some medicinal plants act as self-defence mechanisms to protect the plant against infectious organisms (Tana *et al*., 2015). Local people use medicinal plants in their cultures as flavouring agents and as natural preservatives in food and to treat infection as diseases. For example, in Oromia region, north Shewa zone the local people use medicinal plants including garlic, black pepper, sombo, urgesa, ginger, and damekese to treat various diseases. Base on this information and observation of the practices of the local people to use these plants to treat wound infection, these three medicinal plants were selected. In addition, there is limited scientific information on the medicinal uses of *Ekebergia capensis, Catha edulis and Premna resinos*a as an anti-biofilm, QS, antimicrobial and antifungal properties. Therefore, evaluation of anti-quorum sensing, anti-biofilm and antimicrobial properties of these locally available potential medicinal plants like *Catha edulis, Premna resinosa* and *Ekebergia capensis* are very important. In line with the above information, the aim of this current study was to isolate bioactive compounds from *Ekebergia capensis, Catha edulis* and *Premna resinosa* for anti-biofilm and anti-quorum sensing activities.

1.3. Significance of the Study

Microbial infections are causing great troubles for humanity due to lack of vaccine against some infections, especially pathogenic bacteria, emergence of drug resistance and the resurgence of infection among the others (Barron and Leung, 2015). The disruption/inhibition of biofilms was shown to be effective in reducing hazards usually associated with biofilm formation. There are no universal or ideal biofilm disruptor methods and the search for novel ones is on demand. Plant derived essential oils are well-known antimicrobial agents. Phytochemical of medicinal plants find important application as antimicrobial agents, flavoring agents in food and beverages. Their application as food preservatives to control food spoilage and food borne pathogenic bacteria is due to their growth inhibition property of medicinal plants (Tanver *et al*., 2017).

In Ethiopia, many communities especially from the rural areas still rely on herbal remedies. However, there is little information regarding literature on the use of medicinal plants of Ethiopia. This study contributed to generate information regarding medicinal plants of Ethiopia and accumulating knowledge and methods of biofilms disruption/inhibition.

1.4. Hypothesis

When the people chew *Catha edulis*, they drink water repeatedly and it seems *Catha edulis* has dehydrating property. This dehydrating property might arise from a phytochemical naturally occurring in the leaves of *Catha edulis*. Secondly, the local people were seen while using the root of *Premna resinosa* traditionally for the treatment of tooth infection. After chewing the root of *Premna resinosa* the solution could remain in contact with infected teeth. Thirdly, Local people use bark of *Ekebergia capensis* traditionally for treatment of wound infection. Based on this observation the following hypotheses were developed. Leave extract of *Catha edulis*, root extract of *Premna resinosa* and bark extract of *Ekebergia capensis* might have anti-biofilm, anti-quorum and antimicrobial activity against bacteria and fungi.

1.5. Objectives

1.5.1. General Objective

The general objective of this study was to:

Isolate bioactive compounds from *E. capensis*, *C. edulis* and *P. resinosa* for their antimicrobial's activity test

1.4.2. Specific Objectives

The specific objectives of this study were to:

i. Extract antimicrobial compounds from three medicinal plants using an extraction solvent

ii. Isolate bioactive compounds by column chromatography and elucidate the structures using NMR, IR and melting point

iii. Determine antimicrobial, anti-quorum sensing and antibiofilm activities of extracts and its purified compounds against *S. aureus, P. aeruginosa*, and *C. albicans*

iv. Evaluate lethality and behavioral changes of extracts on leech (*Hirudinaria granulosa*)

2. LITERATURE REVIEW

2.1. Biofilm

Biofilm is a community of microorganisms in which microbial cells attach to each other on a living or non-living surface within a self-produced matrix of extracellular polymeric substance (Bueno, 2014). Biofilms are fixed in a matrix which contains polysaccharides, proteins, and extracellular microbial DNA. Because it provides a reservoir for microbial cells, its dispersion increases the risk of chronic and persistent infections. It may also promote the reinfection of colonized sites (Bueno, 2014). Similarly, the matrix confers a protection against biocides and drugs and have environmental promoters that induce biofilm formation and contributes to drug resistance development (Tan *et al*., 2014).

Different mechanisms have been reported for increased antimicrobial resistance in biofilm structures such as: low diffusion of antibiotics across the polysaccharide matrix. Another mechanism of increasing antimicrobial resistance are: (I) increase of the transmembrane pressure drop, increase of feed channel pressure drop, and increase of transmembrane passage, (II) Physiological changes due to slow growth rate and starvation responses (oxygen, nutrient deprivation or environmental stress), (III) Phenotypic change of the cells forming the biofilm, quorum-sensing, the expression of efflux pumps that decrease intracellular antimicrobial concentration, and (IV) the emergence of persisted cells which are multi drug-tolerant cells that have not acquired genetic resistance (Bueno, 2014).

Likewise, biofilm structure enhances the antibiotic resistance through facilitated horizontal gene transfer due to the high microbial population density. Through conjugation process resistance gene permits biofilm formation (Beceiro *et al*., 2013). All these factors contribute to biofilm cells being 1000-fold more resistant to antimicrobial agents than planktonic cells (Bueno, 2014).

2.2. Biofilm Development

Biofilm formation is a dynamic and complex process influenced by several bacterial and environmental factors (Vogeleer *et al*., 2015)**.** The life cycle of a biofilm has four stages. These are: initial attachment, irreversible attachment, expression and maturation, and dispersion (Vogeleer *et al*., 2015).

Some *Pseudomonas* species required flagellar mediated motility to swim toward a surface and transient attachment. Brownian motion influences bacterial first step of biofilm formation (Kostakioti *et al*., 2013). Bacterial cells formed a dense monolayer at one day and from second to third day, cells began developing gradually from microcolonies to macro-colonies.

The final shape is visible to the naked eye with more than one species of cells. During the early stage of biofilm formation bacterial cells are sheltered by hydraulic shear forces and do not contain channel. Flagella, motility and/or chemotaxis is required for attachment to abiotic surfaces. Flagella facilitate the initiation of biofilm formation and it is necessary to enable a bacterium to reach the surface (Park, and Hahm, 2011).

2.3. Quorum Sensing

Quorum sensing (QS) makes the best metabolic and behavioral activities of bacteria for life in close quarters to achieve cell communication by diffusible chemical signals that impact gene regulation response to biochemical molecules called auto inducers, density dependent behavior (Sifri, 2008). Quorum sensing reflects the cells to activate certain processes. The increased synthesis of the signal molecule lead to regulate genes encoding basic metabolic processes and or genes associated with virulence for Gram positive, Gram negative and *Candida albicans* (Sifri, 2008). The first step in quorum sensing regulation is the production of the quorum sensing molecules by utilizing the intracellular machinery. Secreted molecule remains bound to the bacterial surface or spread to the surrounding environment. In the second step, QS is cell density-dependent signaling, for example, biofilm population in dental plaque is usually more than 700 cells accumulating the molecules outside (Li and Tian, 2012).

This increase is either due to the increase in the number of bacteria, lowering of available area. Impermeable structure near environment with a low-level production of the signaling molecules may also increase the effect of signaling molecules (Li and Tian, 2012). In the third step, when signal molecules exceed a threshold concentration, signal molecules are converted to a specific cellular response (gene expression thought population). Autoinducer/receptor complex bind to DNA by signal activated intracellular receptor. Autoinducer/receptor complex bind to DNA by signal activated intracellular receptor. Finally activated quorum sensing process takes actions (Praneenararat *et al*., 2012). Bacterial quorum sensing systems (Table 2.1) that include the ability to incorporate foreign DNA improve the efficiency of virulence and formation biofilms.

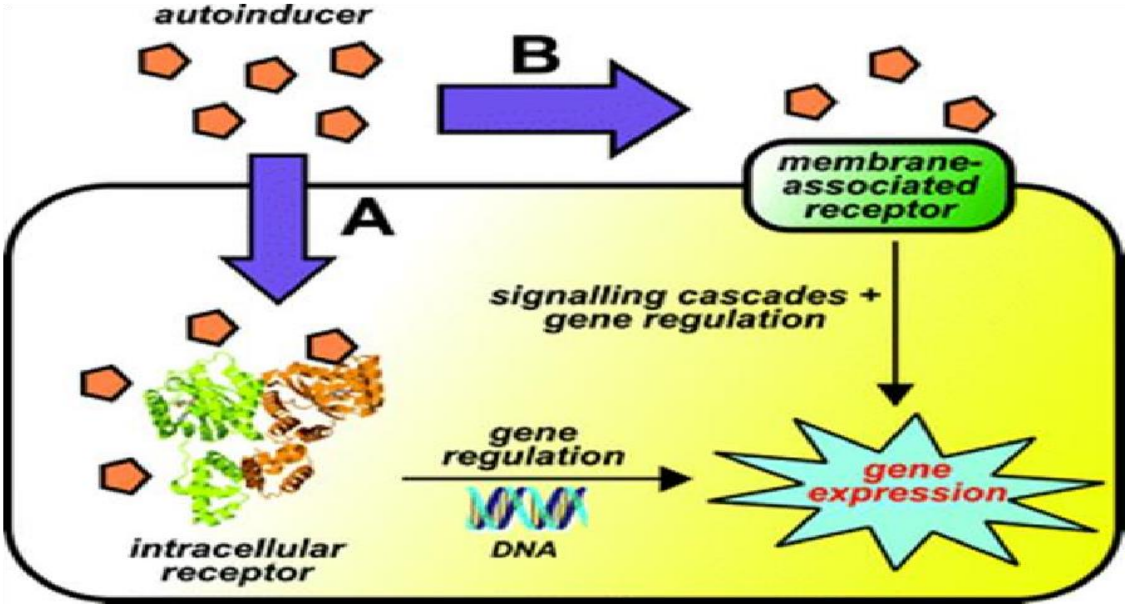


Figure 2.1. Quorum sensing the process of cell-cell communication in bacteria (Praneenararat *et al*., 2012).

Table 2.1. Examples of bacterial quorum sensing systems

Microorganism	Benefits
Bacillus subtlis	Competence, sporulation, biofilm formation, antibiotic production, access of symbionts to nutrient-rich environments in hosts
Pseudomonas aeruginosa	Structured biofilm formation, virulence factors
Staphylococcus aureus	Biofilm formation, virulence factors

(Claverys *et al*., 2006)

2.4. Biofilm Role in Infections

Microscopic evaluation of specimens from chronic wounds often indicates the presence of biofilms. Traditionally, three phases are using to describe wound microbiology. These three phases described as: contamination, colonization, and infection. Contamination refers to the presence of bacteria in the wound, whereas the term colonization is used for bacterial community which is multiplying within the wound but not causing tissue damage. The "critical colonization" is used to describe bacteria that are growing inside the wound but do not possess classical symptoms of infection. However, they adversely affect wound healing (Jamal *et al*., 2015). Bacteria can find its way to a wound from exogenous (soil and water) and endogenous (skin, saliva, urine, and feces) sources. Bacteria that do multiply are not considered "infective" unless and until they pose detrimental effects. This is particularly the case for *Corynebacterial* and *coagulase-negative Staphylococcus* which are considered as commensal skin organisms. However, the multiple microbial population interactions in chronic infections are not completely understood (Jamal *et al*., 2015).

2.6. Test Organisms

2.6.1. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is Gram-negative bacteria, aerobic, rod shape, and motile, have pili (fimbriae), and polysaccharide capsules (Elraih, 2015). It is found in moist places, hospital environment, and disinfectant solution. It can attack not only human, but also a wide variety of hosts (animals, insects, and plants). *P. aeruginosa* causes endogenous infection after three weeks of patient stay in hospitals (Kaiser, 2009).

2.6.2. *Staphylococcus aureus*

Staphylococcus. aureus is Gram-positive cocci, facultative anaerobic bacteria, spherical cells, between 0.5 to 1.7μm in diameter, non-motile and non-spore forming, arranged in grape-like clusters (Figure 2.4). *S. aureus* is the most clinically significant species of *Staphylococci. S. aureus* causes various infections including skin lesions, pneumonia, mastitis, phlebitis, and meningitis (Taha, 2010). *S. aureus* released enterotoxins into food thus causing food poisoning and can release super-antigens into the blood stream leading to toxic shock syndrome (Todar, 2006).

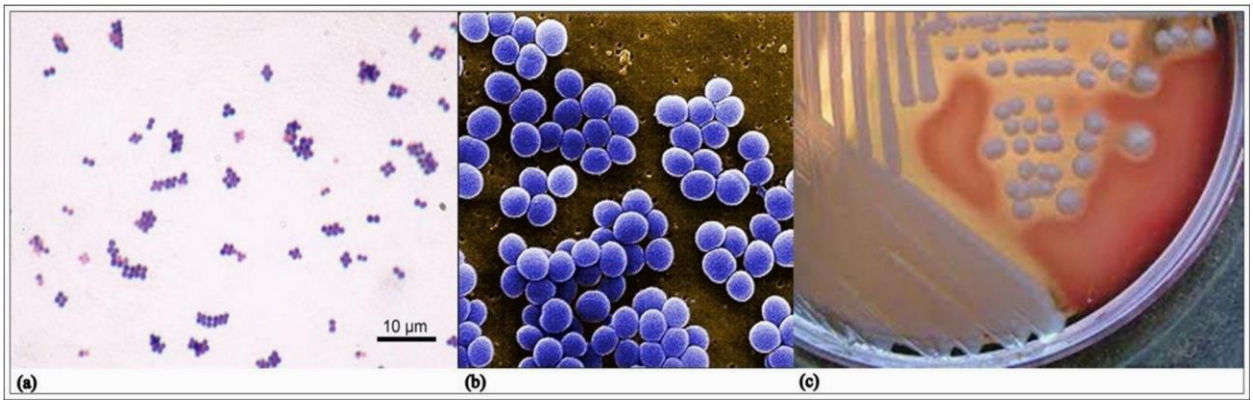


Figure 2.2. Multiple views of *Staphylococcus aureus*

(a) Gram stains of *S. aureus*, (b). Scanning electron micrograph (SEM) of *S. aureus*, (c) colonies of *S. aureus* on blood agar plates showing hemolysis (Buxton, 2013). *S. aureus* expresses many potential virulence factors, inherent and acquired resistance to antimicrobial agents, secreted toxins, exoenzymes immune modulating proteins, and other protein including coagulase.

Its biochemical properties that enhance their survival in phagocytes, protein A, delta-toxin, and leucocidin greatly contribute to its virulence (Todar, 2011). These virulence factors serve many functions; for evasion of host defense mechanisms, for adherence, cell internalization, and for invasion of host tissue.

2.6.3. *Candida albicans*

Candida albicans belongs to the kingdom of fungi in the *Saccharomyceteae* family. According to (Boparai *et al*., 2014) the colonies of *C. albicans* look like cream-colored to yellowish. *C. albicans* is a commensal of the normal human microflora but can also cause a variety of infections in superficial mucosal infections including vagina (Boparai *et al*., 2014). *C. albicans* must cross physical barriers such as epithelial cell layers by active penetration and/or induced endocytosis (Zakikhany *et al*., 2007). *C. albicans* is a part of normal microbiota, normally present in vaginal secretions and discharge and colonizer of human mucosal surfaces. However, under unbalanced conditions, candida growth can become excessive. This is referred to as candida or yeast infection (Zakikhany *et al*., 2007).

2.7. Medicinal Plants as Anti-Biofilm Agents

Natural products were discovered initially because of their therapeutic values in traditional medical practice, but recently, there has been an increasing interest in the biological function and therapeutic roles of natural products and their ecological role in regulating interactions between microorganisms (Krishnan *et al*., 2012). It is speculated that unlike human and mammals that possess immune systems to defend against invaders, plants are lacking such sophisticated immunity toward off invading pathogens, so instead of relying on cellular and biochemical defense systems, plants may have evolved to produce anti-QS compounds that can be used to defeat QS pathogens (Koh *et al*., 2013). Recently, biologically active constituents of natural products, especially plant-derived ones, have led to the discovery of new drugs used for treatment of numerous diseases (Koh *et al*., 2013).

Various studies showed that eukaryotes have evolved efficiently to manipulate bacterial QS systems and protect themselves from pathogen attack. An important discovery was the fact that the halogenated furanones produced by the marine red alga *Delisea pulchra* interfere the *N*-acylated homoserine lactone (AHL) regulatory system in several Gram-negative bacteria. These furanones enable the alga to influence the bacterial colonization and fouling on its own surface in the natural marine environment (Koh *et al*., 2013) Earlier studies have also reported that certain bacteria possess the ability to QS. For example, an *N*-acylhomoserine (AHL) lactonase enzyme, from *Bacillus sp*., was found to hydrolyze the lactone bond of the AHL signaling compound. Interestingly, the paraoxonase (PON) enzymes in human airway epithelial cells also show interference with QS systems (Koh *et al*., 2013).

Table 2.2. Antibiofilm and Anti-quorum sensing medicinal plant against selected bacteria and pathogens.

Source	Antagonist	Inhibition against
Plant extracts	*Melicope lunu-ankenda* (leaves)	*E. coli*
	Syzygium aromaticum (bud)	*P. aeruginosa*
		P. aeruginosa
	Punica granatum (bark)	*C. violaceum*
	P. ginseng (roots)	*P. aeruginosa*
	Lippia alba	*P. putida*
	Zingiber officinale	*E. coli*
	Moringa oleifera (leaves and fruits)	*C. violaceum*
	Laurus nobilis (fruits, flowers, leaves, bark)	*C. violaceum*

(Koh *et al*., 2013)

The mechanism of medicinal plants interactions between quorum sensing microorganism with signal reception, there can be competitive and non-competitive molecules that can interfere with the binding of AHL to its cognate LuxR receptor (Figure 2.6), according to (Koh *et al*., 2013).

Bacteria release AHL molecules which can cause virulence such as biofilms and crown gall formation. The plants, on the other hand, will response to the bacteria signaling molecules by releasing AHL mimic compounds for defense purposes and this will then affect the bacterial QS system.

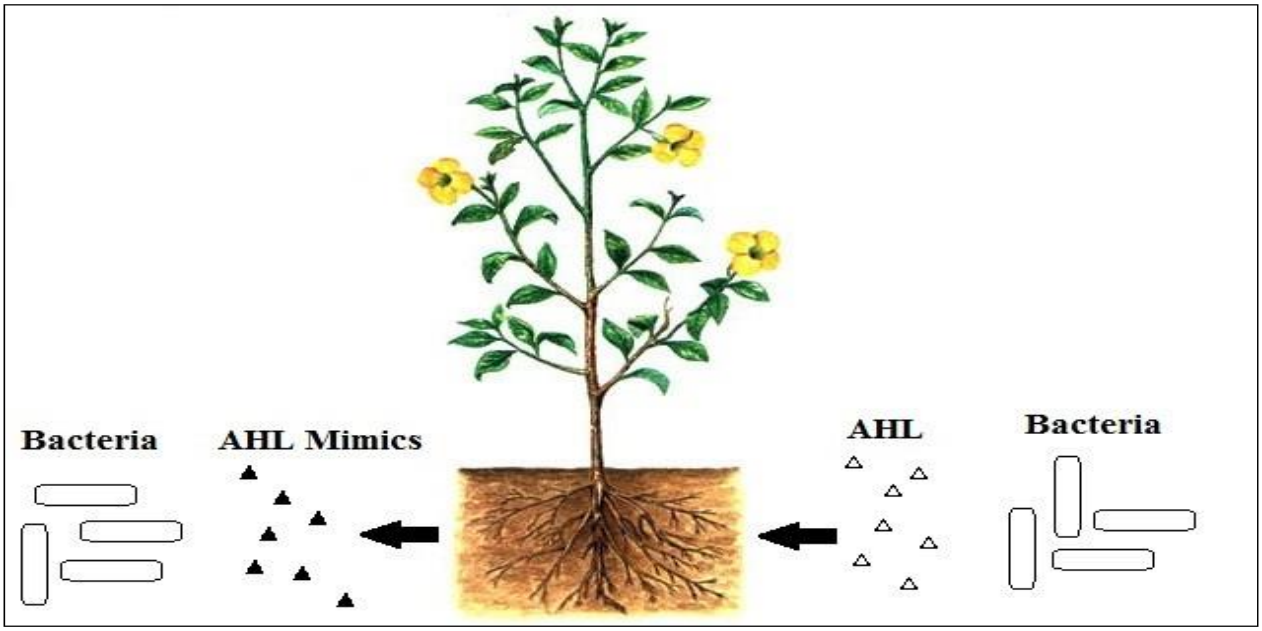


Figure 2.3. The mechanism of *D. pulchra,* grapefruit extract interaction between biofilm and quorum sensing formation microorganism such as in *E. coli, S. typhimurium* and *P. aeruginosa* (Koh *et al*., 2013).

2.8. Experimental Plants

2.8.1. *Catha edulis*

Catha edulis (Jimaa), the edible part that belongs to the score of vegetable materials that humans ingest not for their nutritive value but to experience their psychoactive effects. The young shoots and leaves are the parts chewed for their psychoactive properties. The *Catha edulis* leaves are most commonly 'preserved' for transportation by being wrapped in banana leaves. It contains the alkaloid called cathinone (an amphetamine-like stimulant) and other polyphenolic compound such as tannins and flavonoids, which generally occur as glycosylated derivatives and known for their antioxidant effects (Siddiqui *et al*., 2012). Medicinal value of *Catha edulis* leaves has been used in traditional medicine for the treatment of depression, fatigue, hunger, obesity, and gastric.

2.8.2. *Ekebergia capensis*

Ekebergia capensis locally called as “Somboo” in Afan Oromo. This plant is a large evergreen tree that grows to about 15m in height and occurs in several different habitats. The main stem of *E. capensis* is characterized by a rough light grey to almost black bark, with few buttress roots at the base. The large glossy green leaves that are often tinged with a pinkish patch, or pink edges are pinnate. Pharmacological studies have indicated ant-plasmodial, anti-inflammatory, hypotensive, uterotonic and antituberculotic activities of the crude extracts of this plant. Phytochemical investigations of its stem bark led to isolation of triterpenoids, steroids and flavonoids (Beatrice *et al*., 2014). On other hand the bark of *Ekebergia capensis* were used traditionally for the treatment of wound. The mechanism of using the bark of *Ekebergia capensis* for the treatment of wound were powdered and half a spoon is added to wound.

2.8.3. *Premna resinosa*

Premna resinosa were locally known as “Urgeessaa” in Afan Oromo, is a shrub, 1.5-3m tall comprising of whitish branches and stem. The twigs have anti-resistance properties, while roots are used to make perfumes and as medicine for management of respiratory related illnesses (Ngoci, 2015). The root of *Premna resinosa* were used traditionally for the treatment tooth infection. Based on the information obtained from traditional healers the root of this plant is chewed and the solution can remain on the infected teeth for some time.

3. MATERIALS AND METHODS

3.1. Description of the Study Area

The sample for this study were collected from Oromia Region, North Shewa zone, Kuyu woreda. Kuyu (also known as Gerba Guracha, Afan Oromo: written as Garba Gurraacha) is a town in central part Ethiopia. It is located in the North Shewa Zone of Oromia Region, it has a latitude and longitude of 9°50′0″N 38°20′0″E and an elevation between 2515 and 2547 meters above sea level.

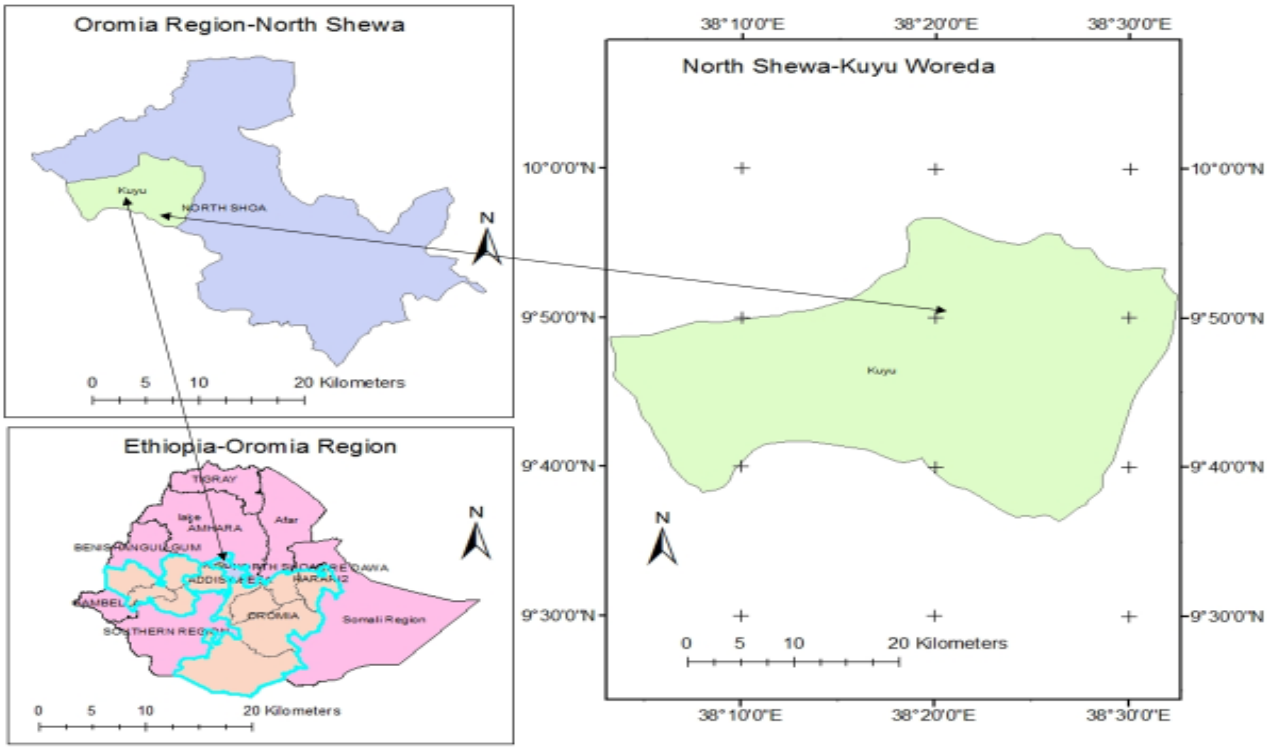


Figure 3.1. Map of study area

3.2. Collection of Experimental Plants

The leaves of *Catha edulis* (1 Kg), (1 Kg) root of *Premna resinos*a and (1 Kg) bark of *Ekebergia capensis* were collected from Oromia Region, North Shewa, Kuyu Woreda. The fresh sample were packed in plastic bags and transported into the laboratory in the month of January 2018. The plant materials were authenticated by a botanist at Biology Department of Addis Ababa University and voucher specimen were stored (Table 3.1) and Figure 3.2).

Table 3.1. Plants, herbarium voucher No and areas of collection

Species (Family)	Herbarium voucher No	Area of collection
E. capensis (Meliaceae)	MG-03/05	Kuyu
C. edulis (Celastraceae)	---------	Adama
P. resinosa (verbenaceae)	EA103	Kuyu

Figure 3.2. Experimental plants photo taken during sample collection (A) *P. resinosa*, (B) *C. edulis* and (C) *E. capensis*

3.3. Preparation of Plant Extracts

The sequential extraction method described by Ncube *et al*., (2008) was applied with slight modifications as follows. The plant samples were washed thoroughly under running tap water to remove dirt, dust and insects and dried under a shade. The finely ground root of *Premna resinosa (*272 g) was soaked in 1.5 L of hexane for 72 hours with occasional swirling to ensure thorough extraction. The soaked materials were filtered, concentrated using rotary evaporator and dried for two days in a bottled in laminar flow hood and weighed. After extraction with hexane, the marc was soaked in 1.5 L of ethyl acetate for 72 hours with occasional swirling to ensure thorough extraction. The extract was filtered, concentrated using rotatory evaporator and dried and weighed. The marc after extraction with ethyl acetate was finally soaked in 1.5 L methanol for 72 hours with occasional swirling. This was filtered, concentrated and

weighed. Sequential extraction procedures as above were repeated with the 273-g bark of *E. capensis* and 245-g leaves of *C. edulis*. The extracts were stored in the freezer at 4°C until tested for antimicrobials, antibiofilm and anti-quorum sensing activities.

3.4. Phytochemical Screening in the Ethyl Acetate Extracts

Detection of 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 (Pandey, and Tripathi, 2014).

Detection of Saponins:

Froth Test: Extracts were diluted with distilled water to 20ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1cm layer of foam indicates the presence of saponins (Pandey, and Tripathi, 2014).

Detection of Tannins:

Gelatin Test: To the extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins (Pandey, and Tripathi, 2014).

Detection of Flavonoids:

Alkaline Reagent Test: Extracts were treated with three drops of sodium hydroxide solution. Formation of intense yellow color, which becomes colorless on addition of dilute acid, indicates the presence of flavonoids (Pandey, and Tripathi, 2014).

Detection of Terpenoids

The powder extract was dissolved in chloroform and placed in Erlenmeyer flask. Then closed with plastic and shake the solution with magnetic stirrer for 2 hours. Formation of intense red color indicates the presence of terpenoids (Pandey, and Tripathi, 2014).

3.5. Preparation of Stock Solutions

The EtOAc extract of *E. capensis* was dissolved in DMSO in two separate vials to furnish 10 mg/mL and 5 mg/mL. This was repeated for *C. edulis* and *P. resinosa* (Table 3.2.).

Table 3.2. The concentration of each stock solutions for different plant

No	EtOAc extract of	Solvent	Concentration mg/ml
1	*Ekebergia capensis*	DMSO	10
			5
2	*Premna resinos*a	DMSO	10
			5
3	*Catha edulis*	DMSO	10
			5

NB: EtOAc= Ethyl acetate and DMSO= Dimethyl sulfoxide

3.6. Preparation of Dried Filter Paper Discs

Whatman filter papers were used to prepare discs approximately 6mm in diameter using a puncher. The prepared filter paper discs were mixed with 10mg/ml and 5mg/ml concentration of extract of experimental plants. Then separately placed in a Petri dish and dried to remove the solvent complete in a hot air oven at 40°C for 160 minutes.

3.7. Test Organisms

The reference strains for human pathogenic bacteria representing Gram-positive (*S. aureus* ATCC 6538) and Gram-negative (*P. aeruginosa* ATCC 9027) bacteria and a pathogenic fungus (*Candida albicans* ATTCC 64550) obtained from Ethiopian Institute of Biodiversity were used for the anti-quorum sensing, antibiofilm properties, and antifungal test against crude extract of experimental plant.

3.8. McFarland Standards Preparation

0.5 McFarland turbidity standards were prepared by mixing 1% of sulfuric acid and 1% of barium chloride to obtain solutions with specific optical densities. To prepare a 0.5 McFarland standard, 2mL of 1.0% $BaCl_2$ and 2mL of 1% H_2SO_4 solution and stirred to maintain a suspension. Well isolate colonies and same type morphology were pick by cotton swap to inoculate to test tube contain 2mL of 1.0% $BaCl_2$ and 2mL of 1% H_2SO_4 solution. 0.5 McFarland turbidity standard provides an optical density comparable to the density of a bacterial suspension 1.5x 10^8 colony forming units (CFU/ml).

3.9. Preparation and Component of Luria Bertani

Table 3.3. Components of LB and Preparation methods

Reagents/Volume	1,000ml
Tryptone	10 g
Yeast extract	5 g
Nutrient Agar	15g
Sodium chloride	5 g

To prepare 1000ml of LB, 10grams of tryptone, 5grams of yeast extract, 15grams of nutrient agar and 5grams sodium chloride were weighted and added in conical flask. 1000ml Distell water was added and mixed. To dissolve all ingredients completely, boiling while stirring. After completely dissolved it was sterilized by autoclaving (15 minutes) from 121-124°C on liquid cycle).

3.10. Antimicrobial Susceptibility Test

The antimicrobial activity test of the crude extracts of experimental plants against one Gram-positive (*S. aureus)* and Gram-negative bacteria (*P. aeruginosa) and Fungus (C. albicans)* were carried out by disk diffusion method. The MHA and SDA were prepared for each organism as follows. 20ml of sterile MHA (maintained at 45-50^{o}C in a molten state), and SDA poured into sterilized Petri dishes separately and set aside. After solidifying, 0.5McFarland standard of test organisms were spread on MHA plate and fungus were spread on SDA. Then

previously prepared filter paper discs were place onto the surface of the agar plate at equal distance from each other and 15 mm from edge of plate as described by (Clutterbuck *et al.*, 2007). Each disc was pressed down to ensure complete contact with the agar surface and the discs were placed with a dispensing apparatus, they distributed evenly. Each plate was inverted and placed in an incubator set at 37^0c for 24 hours after the discs are applied for bacteria. The antimicrobials susceptibility was evaluated by measuring zone of inhibition using plastic ruler in mm.

3.11. Antibiofilm Activity Test

3.11.1. Test Tube Method

Test organisms (0.1mL) obtained by adjusting turbidity to 0.5McFarland standards was transferred to test tubes containing 10mL LB. Two hundred microliter of crude extract of each of the experimental plants 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. All tests were carried out in triplicates and results of the test were recorded.

3.11.2. Swarming Motility Test

Swarm plates were prepared using nutrient agar containing 0.5% peptone, 0.2% yeast extract and 1.0% glucose per 100ml of distilled water. 250µl of plant extracts were added to 10ml semisolid agar, gently mixed and poured immediately onto the surface of a 10ml of pre-warmed agar plate as an overlay. Twenty microliters of standardized inoculums were placed on the center of the plate. Similar volume of test organism was placed on the center as negative control plate (overlay agar without extract) and the plates were incubated for overnight at 37^{o}C upright position for 30h.

3.12. Anti-Quorum Sensing Activity Test

3.12.1. Flask Incubation Test for Quantification of Violence in Production

Two hundred microliters of test organism were inoculated in Erlenmeyer flasks containing 20ml of LB supplemented with crude extract of experimental plants 5mg/ml and 10mg/ml concentrations. The flasks were incubated at 37°C in a shaking incubator for 30hr. Two milliliters of each culture were transferred to test tube and centrifuged at 1500 rpm for 15min

to precipitate the insoluble pigment. The pellet was re-suspended in 2ml of dimethyl sulfoxide (DMSO) and homogenized by vortexing. Violacein absorbance at 585nm was determined using UV–visible spectrophotometer. The negative control sample consisted of incubating the test organism in LB broth without adding extract and violacein absorbance was determined with the same procedure of test samples. Inhibition was calculated with respect to control and triplicate measurements were performed.

3.13. QS Inhibitory Effect of Extracts on Pasteurized Milk

Twenty milliliters of pasteurized milk were inoculated with twenty micro-liters of *P. aeruginosa*. Samples inoculated with the *P. aeruginosa* were grown in the absence and presence of extract of our experimental plants. The samples were incubated at room temperature for a period of 48h. The quorum sensing inhibitor effect of crude extract were observed.

3.14. Lethality and Behavioral Changes of Leeches by Crude Extracts

The lethality and behavioral changes study of leeches by extracts was performed on leech by using a method developed by (Nguta *et al*., 2013) with slight modification. Five hundred milliliter of fresh lake water was taken in chamber and ten stone (4.6 to 6 gm) were added. The chambers were filled with 500ml of fresh lake water. Larvae of leech was placed in the chamber and algae were added to act as food and produce oxygen for the larvae of leech.

Various mass of the extract (100, 250 and 500mg) were added to fresh lake water in the chambers. Ten leeches were drawn from the fresh lake waters and placed in each chamber. Control group was done using fresh water only in the case of comparing the number of died and number of lived in test group in each time. Three replicates for the three mass of different crude extracts and the control were performed. Lethality and behavioral change of larvae were checked in span of two hours using a magnifying glass and the average mortality at each concentration were determined as it may be essential for estimation of lethal dose (LD_{50}).

Figure 3.3. Leeches collected from Andode lake (A) Andode lake, (B) Chamber of test group, (C) Collected leech on hand and (D) Chamber of control group.

3.15. Isolation of Bioactive Compounds

3.15.1. Column Chromatography

The most active ethyl acetate extract of *E. capensis* was subjected to silica gel column chromatography. Briefly, silica gel (130 g) was mixed with 200mL of n-hexane 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. The EtOAc extract (2.5 g) was adsorbed on equal amount of silica gel and subjected to column chromatography using hexane:EtOAc:MeOH of increasing polarities as eluent to give 80 fractions. Each 75 mL was collected. The column was first eluted with n-hexane as the mobile phase with the polarity increasing by 5 % increments of ethyl acetate. After getting to 100 % ethyl acetate, the polarity was further increased by 5 % increments of methanol.

3.15.2. Structure Elucidation of the Bioactive Compounds

Analytical thin layer chromatograms were run on a readymade 0.2 mm thick layer of silica gel GF254 (Merck) coated on aluminium plate. Column chromatography was performed using silica gel (230-400 mesh) Merck. ^{1}H-, ^{13}C- and DEPT-NMR spectra were recorded on a Bruker advance 400 spectrometer operating at 400 MHz in $CDCl_3$ and DMSO-d_6. All chemical shifts were stated in parts per million (ppm) and coupling constants were expressed

in Hz. Infrared (IR) spectra were obtained on Perkin-Elmer 65FT ((IR ν_{max} KBr (4000-400) cm^{-1}) infrared spectrometer using KBr pellets.

3.15.3. Measurement of Melting Point

Melting point of the extracted bioactive compound was measured by using a melting point apparatus (Thiele tube). The sample (1mg) was inserted into a glass capillary tube and placed in the aluminium block of the sample chamber. The block was heated slowly (2°C per min) and the sample was observed through the magnifying lens until melting was observed. The melting point temperature was recorded from the light-emitting diode (LED) display.

3.16. Data Analysis

The data was analysed using Minitab (Version 17) software's and Excel Microsoft Word. The differences between test groups and control groups were analysed by one-way and two-way analysis of variance (ANOVA) and the results were presented using tables and graphs in form of mean ± SEM (standard error of the mean). A p-value of less than 0.05 was considered statistically significant at 95% level of confidence.

Summary of antimicrobials, antibiofilm, anti-quorum sensing extraction and bioactive compound isolation process are shown in Figures 3.4 and 3.5 respectively.

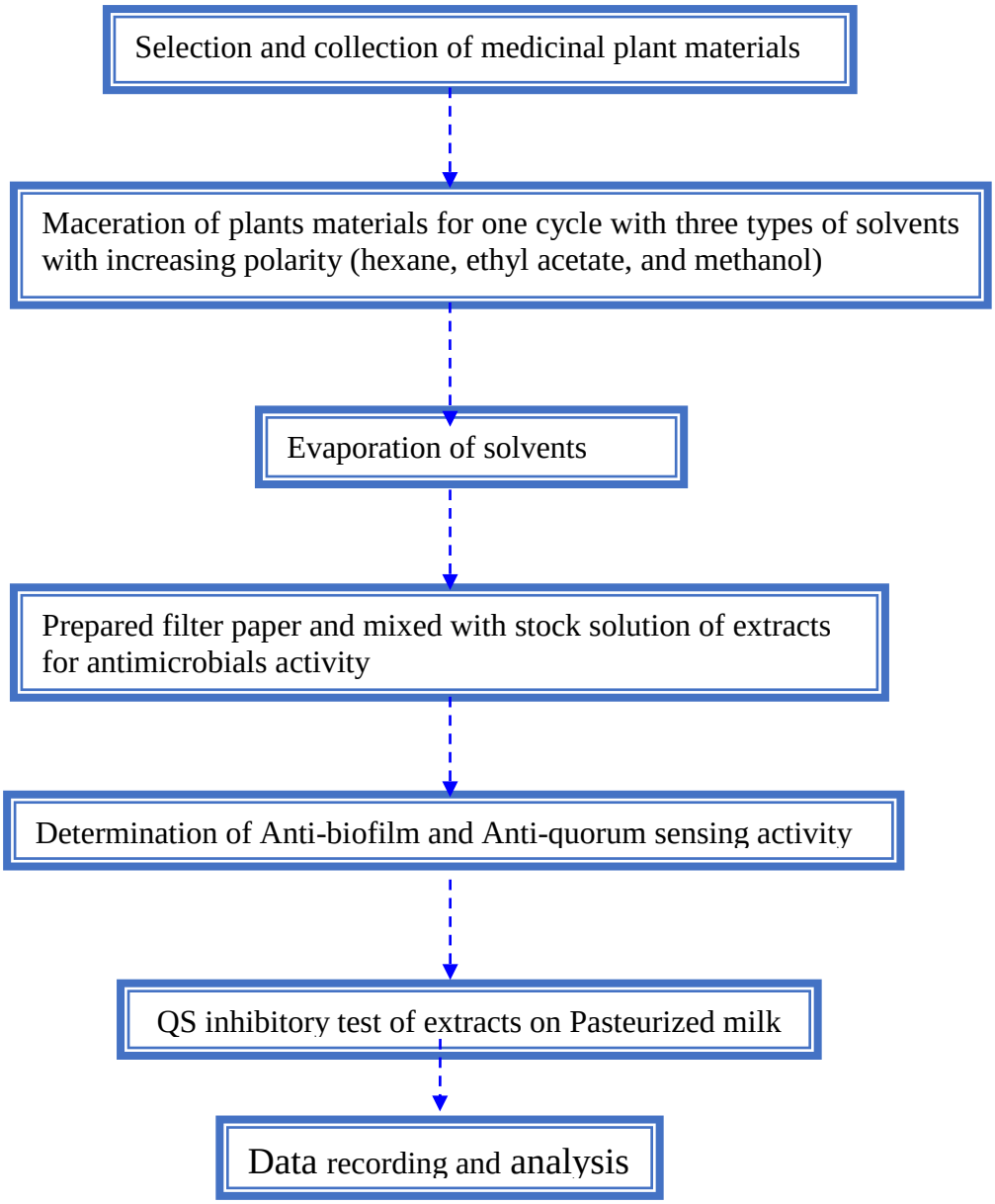


Figure 3.4. Methodology flow diagram of Antimicrobials, Antibiofilm, Anti-quorum Sensing

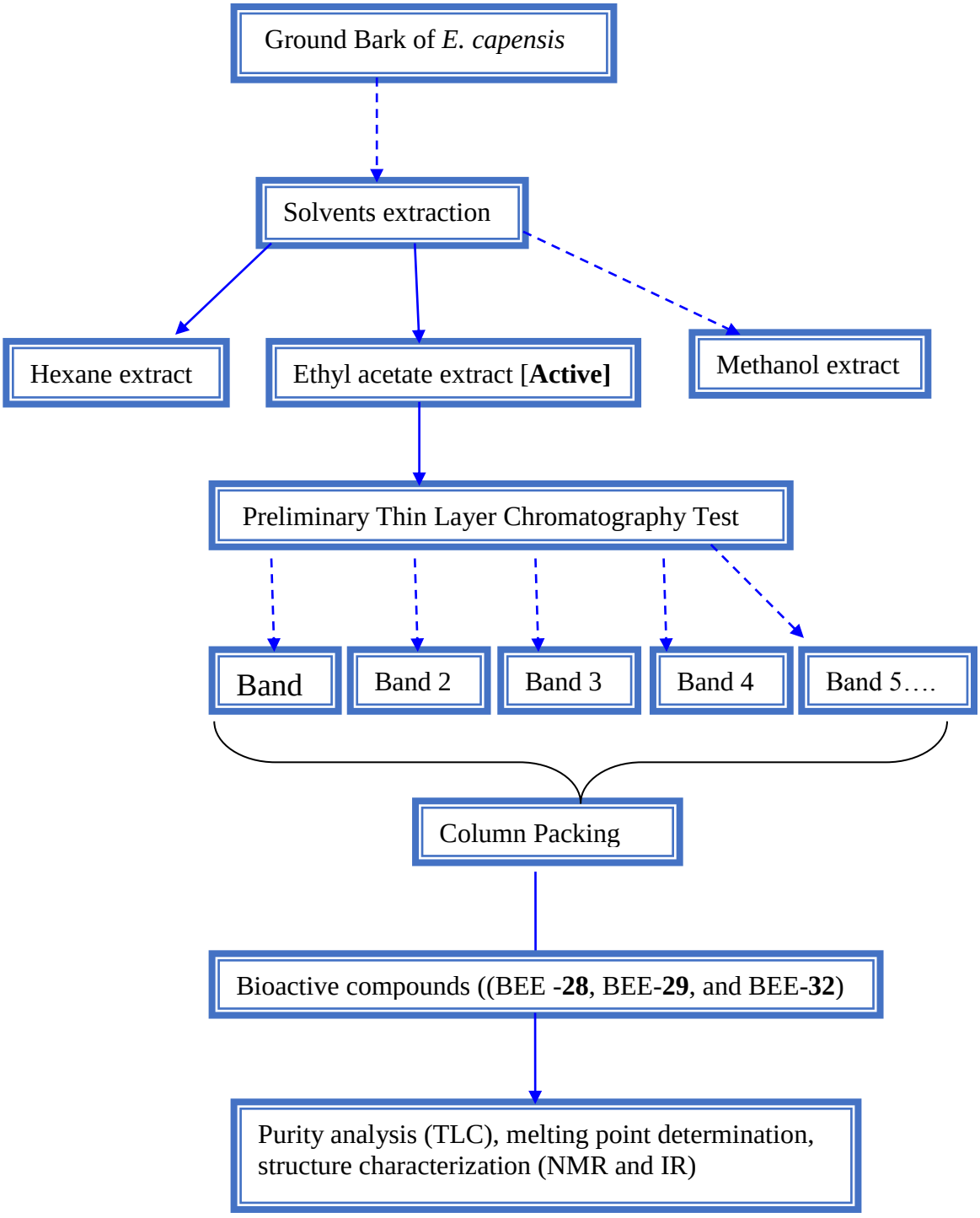


Figure 3.5. Flow chart of the bioactive compounds extracted and characterization from ethyl acetate extract bark of *E. capensis*.

4. RESULTS

4.1. Yield of Extracts

The highest percentage yield was extracted from *E. capensis* by ethyl acetate which was 1.72% followed by methanol extract of *Catha edulis* and *Premna resinosa* 1.62% and 1.01% respectively. On the other hand, the lowest percentage yield of 1.13%, 1.17%, and 0.17% was obtained from hexane extract of *Catha edulis, Ekebergia capensis and Premna resinosa* respectively (Figure 4.1 and Appendix B).

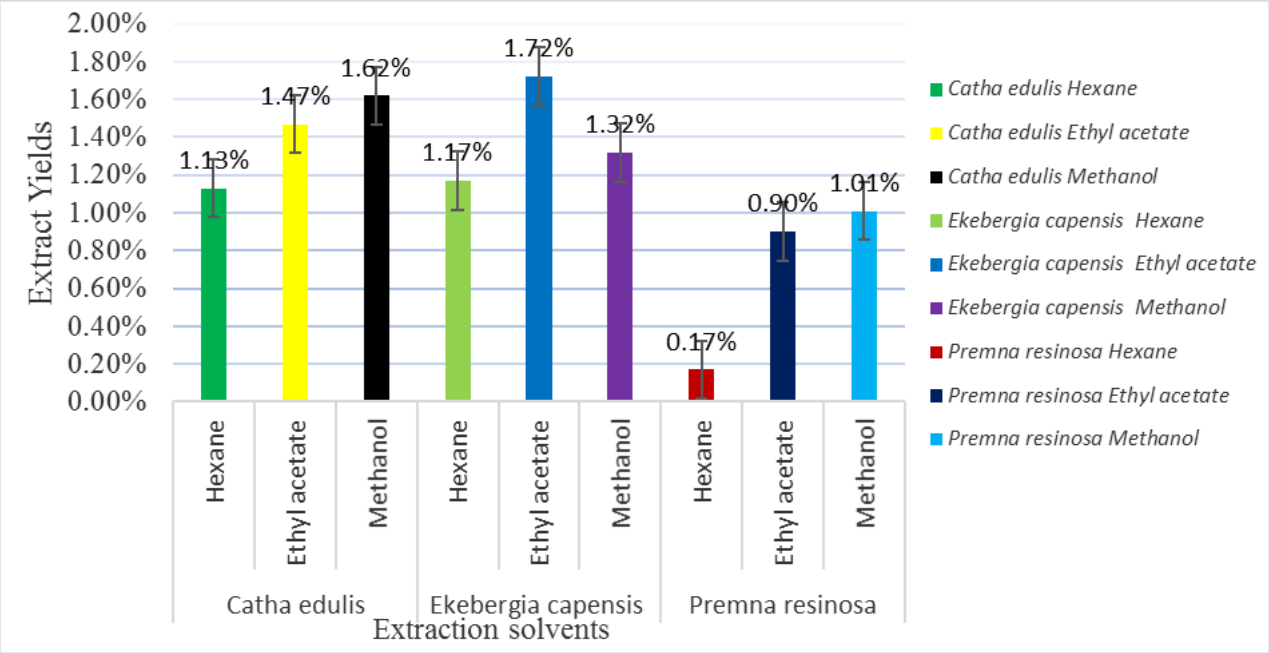


Figure 4.1. Percentage of yields of extracts obtained from the leaves of *Catha edulis*, bark of *E. capensis* and root of *Premna resinosa*.

4.2. Phytochemical Screening

Tannin was present in all the crude extracts while *Ekebergia capensis* ethyl acetate extracts have alkaloids, saponin, tannin, terpenoids and flavonoids however, only saponin and tannin were obtained from *Premna resinosa* in this study (Table 4.1).

Table 4.1. Presence of alkaloids, saponin, flavonoids, tannin and terpenoids in extracts of experimental plants

Extracts	Alkaloids	Saponin	Tannin	Flavonoids	Terpenoids
Catha edulis	+	_	+	+	_
Premna resinosa	_	+	+	_	_
Ekebergia capensis	+	+	+	+	+

NB: - absence, + presence

4.3. TLC Profile of the Extracts

After extractions, all extracts obtained from the three plants were analysed with TLC. Results obtained revealed that the ethyl acetate extract bark of *Ekebergia capensis* contains many spots which were visualized after spraying with vanillin compared to hexane and methanol extracts (Figure 4.2 and Appendix C).

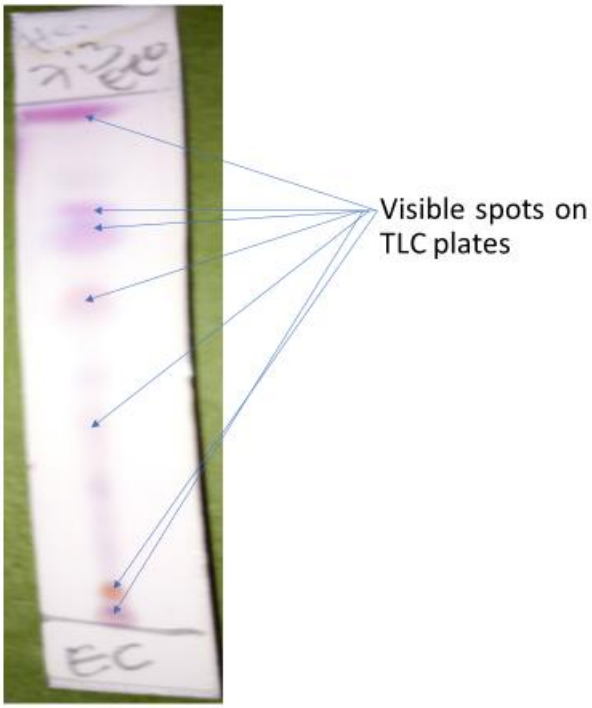


Figure 4. 2. Visible spots on TLC plates obtained from ethyl acetate extract of *E. capensis*

4.4. Antimicrobials Activity of Extracts

The ethyl acetate crude extracts of *E. capensis* showed antibacterial activity on *S. aureus* and antifungal activity on *C. albicans* at 10mg/ml concentrations. The results revealed that *E. capensis* crude extract inhibit the growth of *S. aureus, C. albicans* but did not inhibit growth of *P. aeruginosa* (Table 4.2 and Figures 4.3).

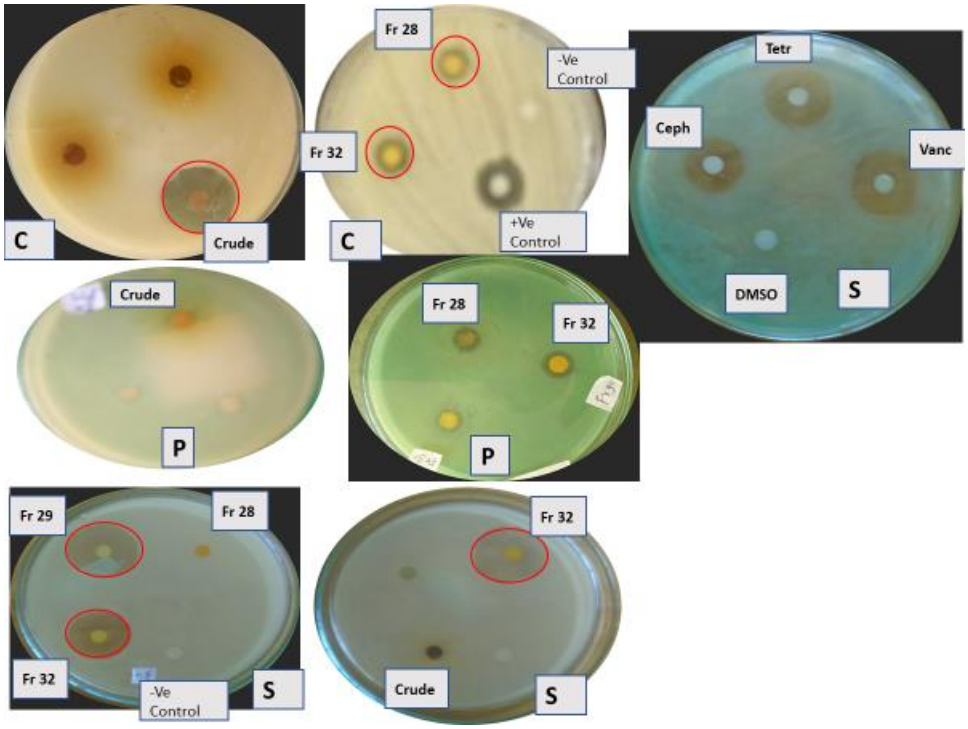


Figure 4.3. Antimicrobial activities of extract of *E. capensis*, *C. edulis* and *P. resinosa* against *C. albicans*, *S. aureus* at 10mg/ml concertation. (C) *C. albicans*, (P) *P. resinosa*, (S) *S. aureus*, (Fr) fractionated compounds, (Ceph) Cefalexin, (Tetr) tetracycline, (Van), Vancomycin and circle shows diameter of inhibition zone.

4.2. Effect of ethyl acetate crude extract of *E. capensis, P. resinosa* and *C. edulis* at 10mg/ml against *S. aureus, P. aeruginosa, C. albicans*.

No	Plant species	Extracted by	Zone of inhibition in mm		
			S. aureus	*P. aeruginosa*	*C. albicans*
1	*Ekebergia capensis*	Ethyl acetate	16±1.5	NI	20±1.98
		n-hexane	NI	NI	6.5±0.34
		Methanol	6±1.22	NI	4±0.67
2	*Premna resinosa*	Ethyl acetate	NI	NI	NI
		n-hexane	NI	NI	NI
		Methanol	NI	NI	NI
3	*Catha edulis*	Ethyl acetate	4.5±0.54	NI	NI
		n-hexane	NI	NI	NI
		Methanol	6.5±1.02	NI	8±0.22

The ethyl acetate extract of *E. capensis* showed highest degrees of antibacterial (*S. aureus*) and antifungals (*C. albicans*) activity ranged between 4-20mm inhibition zone. Even though extracts root of *P. resinosa* does not show activity against *P. aeruginosa*, *C. albicans* at 10mg/ml (Figure 4.3). The largest zone of inhibition was observed for *E. capensis* (ethyl acetate extract) with inhibition zone of 20mm for *C. albicans,* 16mm for *S. aureus.*

Premna resinosa extract showed no inhibition zone at various concentrations against all the teste organisms. However, largest zone of inhibitions was observed for ethyl acetate extract of *E. capensis at* 10mg/ml concentration for all tested microorganisms except *P. aeruginosa.*

The bioactivity results were considered evidence of the presence of more compounds in the crude extract of ethyl acetate, compared to the methanol and hexane extracts of *Ekebergia capensis*, the probable reason being that the compounds in bark of *E. capensis* were more soluble in ethyl acetate as compared to methanol and hexane. However, different activities against *S. aureus, P. aeruginosa, C. albicans* were observed by fractionated compounds. The inhibition activity at different concentration showed different zone of inhibition by purified compounds from ethyl acetate extract of *E. capensis* (Figure 4.3). This purified compound from ethyl acetate extract of *E. capensis* used in this study showed variable antibacterial activities against *P. aeruginosa* at concentrations ranging from 10 to 5mg/ml.

4.5. Anti-Biofilm Activity Test

4.5.1. Test Tube Method

Strong biofilm formation by *C. albicans* and *P. aeruginosa* compared with control as observed by the heavy crystal violate pigment remaining on the test tube. The other such as *S. aureus*, and laboratory isolate bacteria (A and B) were a little biofilm formed when they treated with ethyl acetate extract of *E. capensis*. Figure 4.4 is showing the adherent of biofilm stained by crystal violate. The initial cell attachment significantly inhibited in case of *E. capensis* against *S. aureus* and Isolate (A and B) at 10 mg/ml concentration.

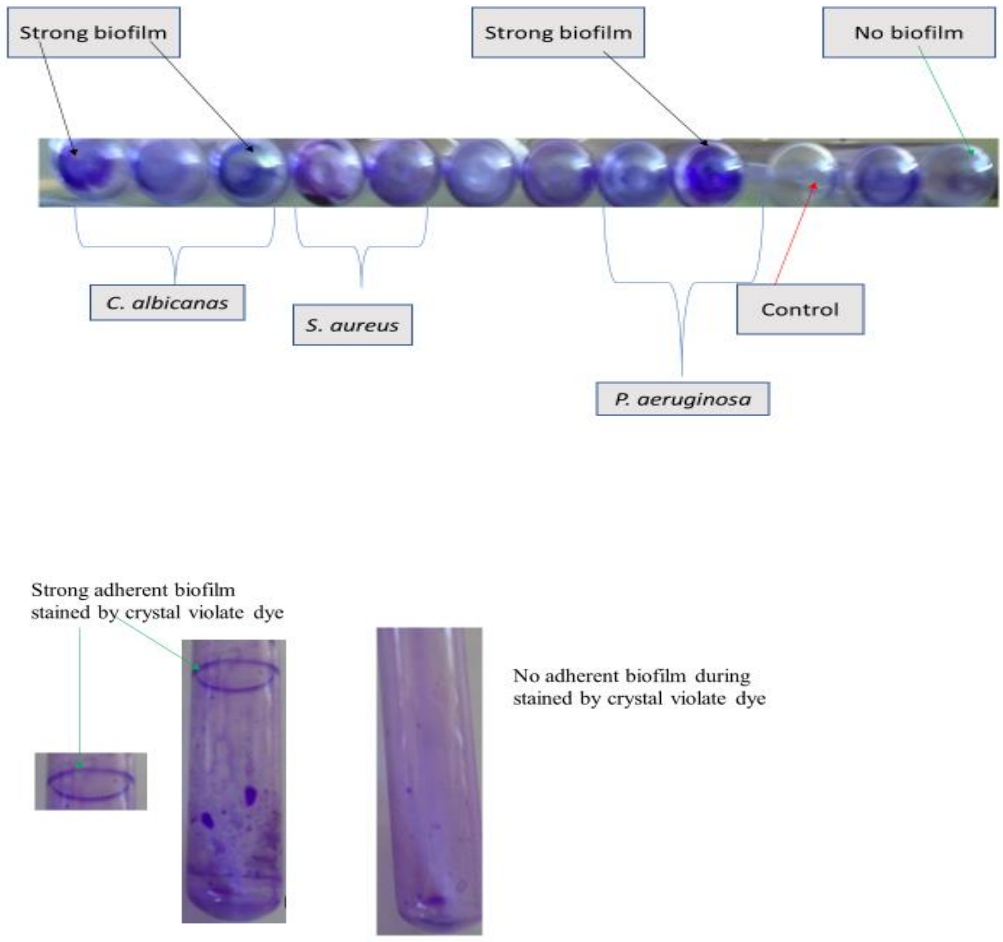


Figure 4.4. Anti-biofilm activities against *C. albicans*, *P. aeruginosa*, *S. aureus* crystal violate staining method.

The crystal violet assay indicated that the effect of *E. capensis* extract on initial cell attachment. To ensure a concentration that is affecting the microbial attachment, concentrations at 10mg/ml and 5mg/ml for antibiofilm assay were used. Crystal violate staining method is easily and widely used to measure both the formation and inhibition of biofilms. Ethyl acetate extract of *E. capensis* at concentration of 10mg/ml is the lowest concentration, which inhibited on *S. aureus* biofilm formation.

4.5.2. Swarming Motility Inhibition

The swarming motility assay, overnight cultures of the test bacteria were point inoculated at the centre of the medium consisting of 1% tryptone, 0.5% NaCl and 0.3% agar with 10mg/ml concentrations of extracts. The plates were incubated at 30 °C in upright position for 24h. Following swarm zones of the bacterial cells swarming migration were observed (Figure:4.5). Figure 4. 5 indicates that ethyl *acetate* extract of *E. capensis* has significant influence on the swarming motility but concertation different activity among different microorganisms. Best anti-swarming activities were shown by 10mg/ml of extract on *P. aeruginosa*, *S. aureus and* Isolate B. But in the same extract concentration (10 mg/ml) Isolate A showed less swarming inhibition activity.

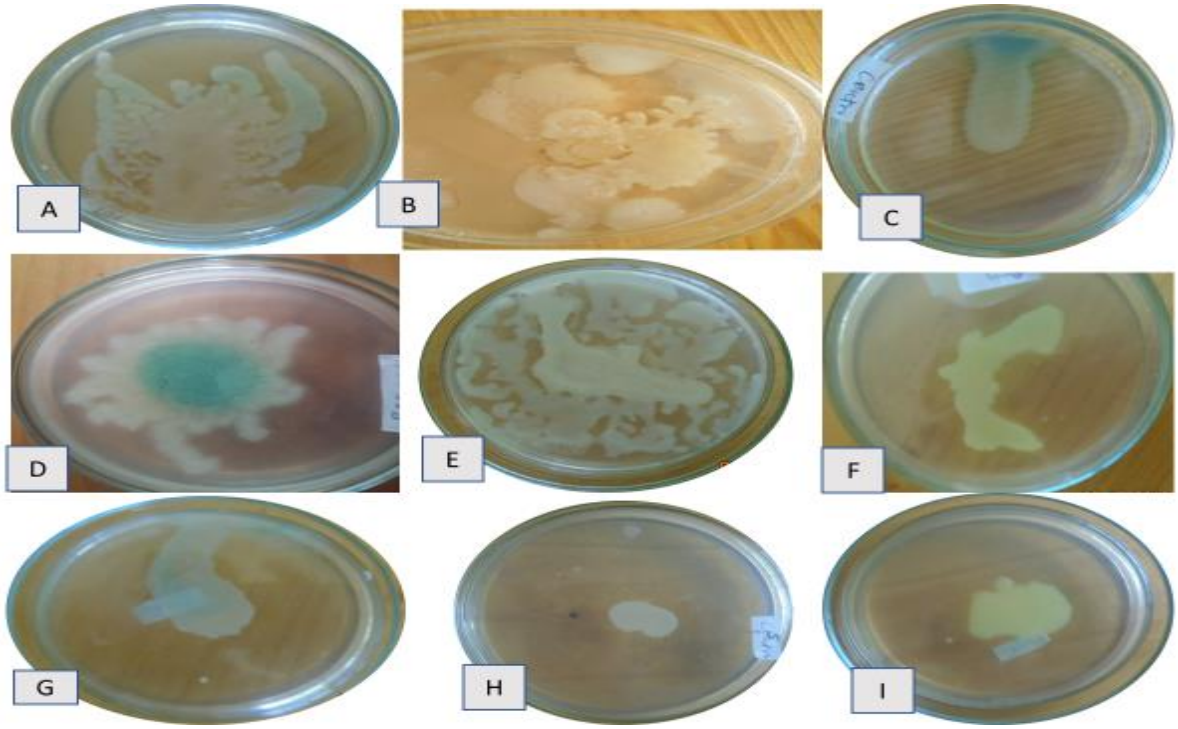


Figure 4.5. The migration distance of *S. aureus, P. aeruginosa C. albicans* was observed while they were incubated for 24 hours at 10mg/ml concentrations of *E. capensis* extract and control: without extract. (A) *P. aeruginosa,* (B) *S. aureus,* (C and G) *C. albicans,* (F and I) control *P. aeruginosa (*H) control *S. aureus*

4.6. Anti-quorum Sensing Assay

4.6.1. Flask Incubation for Violacein Inhibition Production

Inhibition of purple pigment by *S. aureus,* and *C. albicans* on the presence of extract of *E. capensis* are an indication of quorum sensing inhibition. The inhibitory effect of the *E. capensis* extract on violacein pigment production was measured and quantified by spectrophotometers (Table 4.3 list of absorbance measurements). A concentration of 10mg/ml extract inhibited about 72.6 % violacein production which has about 4 times higher inhibition than 5mg/ml concentrations inhibition (16.2% inhibition). This value was determined by the following formula:

$$\text{Inhibition of violacein production} = \frac{\text{Absorbance of control - Absorbance of sample}}{\text{Absorbance of control}} \times 100\ \%$$

Table 4.3. Inhibition of violacein production by the crude extract against *P. aeruginosa, S. aureus*, and C. *albicans.*

	Plant species	Microorganisms	**Concentration mg/ml**		% of inhibition violacein production at 10mg/ml
			5	**10**	
			Absorbance at 585nm		
1	*E. capensis*	*P. aeruginosa*	0.55	0.20	67.74%
		S. aureus	0.52	0.17	72.6%
		C. albicans	0.57	0.19	69.35%
2	*C. edulis*	*P. aeruginosa*	0.57	0.49	21%
		S. aureus	0.56	0.32	48.4%
		C. albicans	0.58	0.39	37.1%
3	*P. resinosa*	*P. aeruginosa*	0.59	0.51	17.74%
		S. aureus	0.53	0.37	40.32%
		C. albicans	0.58	0.46	25.80%

NB: the absorbance of control was 0.62, since the control have Luria Bertain and 20 microliters of test organisms, but test sample contain Luria Bertain + 20 microliters of test organism +5 and 10mg/ml of crude extract respectively.

4.7. QS Inhibitory of Extracts on Pasteurized Milk

Quorum sensing inhibitory activity of each crude extract of our experimental plants were determined in pasteurized milk. The untreated control and treated with crude extract of *P. resinosa* and milk inoculated with *P. aeruginosa* exhibited spoilage, whereas, milk inoculated with the test organism and treated with a crude extract of *E. capensis* showed minimal spoilage after 48h at room temperature (Figure 4.6).

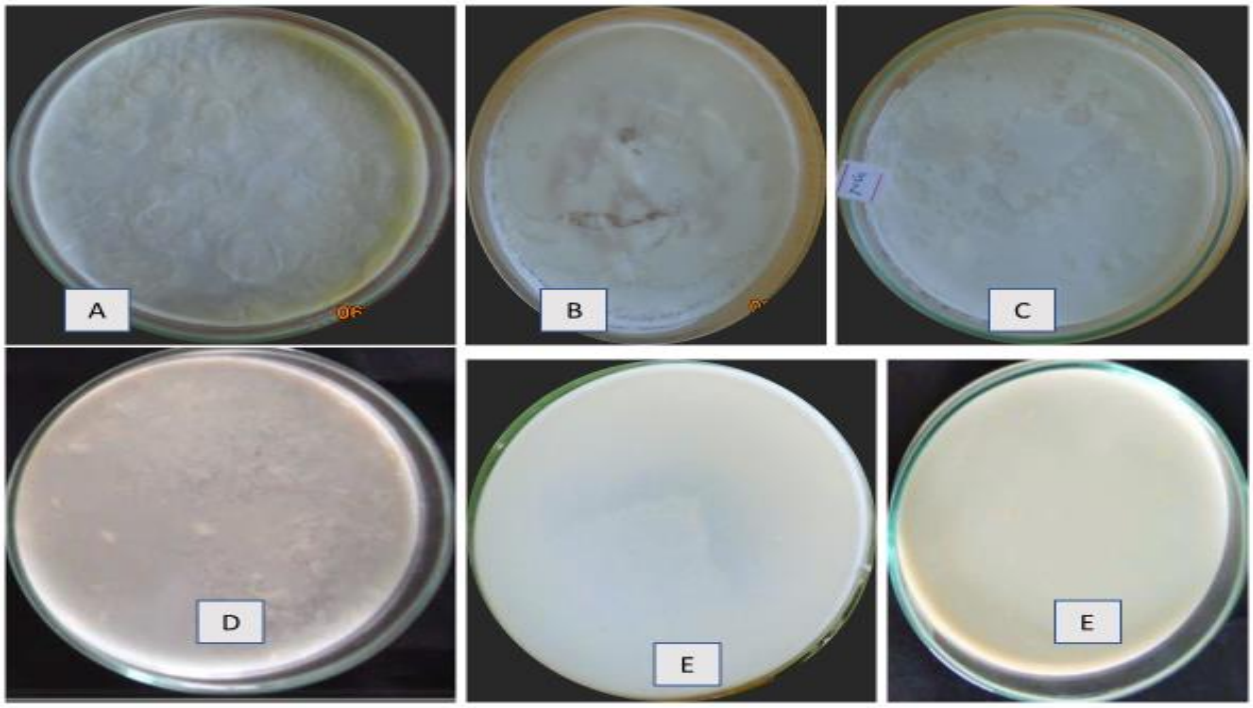


Figure 4.6. *Q*uorum sensing inhibitory activity of extract of *P. resinosa, E. capensis and C. edulis* on pasteurized milk.

A), Control, milk inoculated with *P. aeruginosa* exhibiting spoilage, (B and C), milk treated with crude extract of *C. edulis* exhibited little spoilage, D), milk treated with crude extract of *P. resinosa* exhibited spoilage, E and F), milk treated with crude extract of *E. capensis* inhibited spoilage. As seen from figure 4.8 ethyl acetate extract of *E. capensis* can prevent spoilage of milk by the *P. aeruginosa*. It is well known that spoilage of milk by *Pseudomonas* species is due to their ability to produce the exoenzymes lipases and proteases. This study has shown that the exoenzyme production in gram-negative spoilage bacteria such as *Pseudomonas* species is under QS regulation.

4.8. Lethality and Behavioral Analysis of Extracts on Leeches

Each chamber was examined in every fifteen minutes then every thirty minutes until all the leech were dead or no movements were observed after different concentration extract of the experimental plants were applied separately. The tested sample was considered ineffective if any modification of *leech* behavior were observed in a span of two hours. Survivors were counted, after specified time interval, and the percent deaths at each concentration and control was determined. In cases of death in control chamber, the percent of deaths were calculated by using the following formula:

$$LD_{50} = \frac{\text{Number of test organisms - Number of died}}{\text{control}} \times 100$$

The lethality and behavioural change effect of ethyl acetate extract of *E. capensis*, *Catha edulis* and *P. resinosa* on the leech shown, *E. capensis, Catha edulis* have biological active compounds (Table 4.4). There were changes of movements and their attachments among the ethyl acetate extract of *E. capensis* and *C. edulis* during the observation period but not any changes for *Premna resinosa* crude extracts comparing with control group**.**

Table 4.4. Effect of ethyl acetate extracts of *E. capensis, C. edulis* and *P.* resinosa on leeches

Observation	Control Group				Test Group			
					500 mg extract of *E. capensis*			
	15min	30min	60min	90min	15min	30min	60min	90min
Movement	Normal	Normal	Normal	Normal	Increase	Decrease	No movement	Died (6)
Attachment	Normal	Normal	Normal	Normal	No attached	No attached	No attached	No attached
					500 mg extract of *C. edulis*			
	15min	30min	60min	90min	15min	30min	60min	90min
Movement	Normal	Normal	Normal	Normal	Rapid	Slow	No movement	Died (9)
Attachment	Normal	Normal	Normal	Normal	No attached	No attached	No attached	----
					500 mg extract of *P. resinosa*			
	15min	30min	60min	90min	15min	30min	60min	90min
Movement	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Attachment	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal

NB: Control group (treatment without crude extract); Test group (treatment with 500 mg extract) and the number in the bracket shows the number of died leech from the total of ten, even though the experiments were carried out at 100mg, 250mg, but it takes long period of to kill the leeches

4.9. Isolation and Characterization of Bioactive Compounds

Among the extracts obtained from the three medicinal plants, the most bioactive extract was the ethyl acetate extract of *Ekebergia capensis.* Bioactivity-guided fractionation was performed using column chromatography technique and thin layer chromatography (TLC) to isolate bioactive compounds for antimicrobials, antibiofilm and anti-quorum sensing. Because of ethyl acetate crude extracts of *Ekebergia capensis* plants have shown a higher extract yield and showed the

stronger antimicrobials, antibiofilm and anti-quorum sensing activity. Hence subjected to further fractionation. Each extract was analysed with TLC and fractions having similar Rf value were combined. Among the fractions collected, Fr-32 was collected with 60:40 while Fr-28 and Fr-29 were collected with 70:30. These pure fractions were then subjected to antimicrobials, antibiofilm and anti-quorum sensing analysis. Results of the bioassay showed positive for antimicrobials, antibiofilm by exhibiting their growth and disrupted cell to cell communication as shown by (Figure 4.3).

4.9.1. Compound BEE-28

Compound BEE-28 was isolated as purple solid, melting point at 77-78°C. The infra-red (IR) spectrum of compound BEE-28 (Figure 4.11) showed several intense bands in the region of 4000 and 500 cm^{-1}. The band due to O-H stretching is evident at 3445 cm^{-1}, C-H stretching at 2902 cm^{-1}, and carbonyl group (C=O) at 1710 cm^{-1} and the band at 3000 cm-1 is accounted to C-H stretching of hydrocarbons (Figure 4.10).

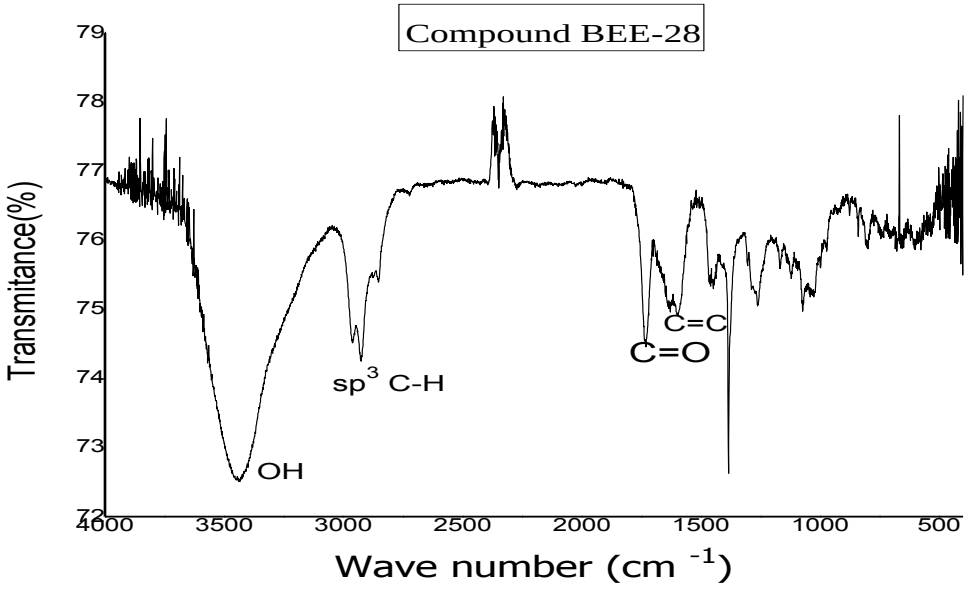


Figure 4. 7. IR spectrum of compound BEE-28. (BEE-Bark of *E. capensis* ethyl acetate extracts)

The ^{1}H-NMR spectrum of compound **28** (Figure 4.9) shows the presence of proton signals in the aliphatic and aromatic region. The doublet signal at δ 0.99 integrating for twelve hydrogens is accounted to the presence of four methyl groups in the compound. Also observed is the signal at 2.06 (2H, sep.) due to the methine proton on carbon bearing two methyl groups. The proton signal on oxygenated carbon is evident at 4.11 (4H, *d*, J = 7.6 Hz). Also observed were signals in the aromatic region at 7.75 (2H, *dd, J* = 9.2 and 3.6 Hz) and 7.56 (2H, *dd, J* = 9.2 and 3.6 Hz).

The ^{13}C-NMR spectrum of compound 28 with the aid of DEPT-135 (Figure 4.13 and Table 4.5) showed the presence of eight carbon resonances of which two quaternaries, three methine, two methyl and one methylene. The carbon signal at δ 167.6 is accounted to the presence of carbonyl carbon of an ester which agrees well with the IR spectrum of the same compound. The three carbon signals in the aromatic region were evident at 132.4, 130.9 and 128.8 ppm. The signal due to an oxygenated methylene carbon was observed at 71.8 ppm. The ^{13}C-NMR spectrum of compound 28 also showed signal in the aliphatic region at 27.7 and 19.1 ppm accounted to the presence of methine and methyl groups, respectively. The data generated agrees with the compound whose structure is shown in Figure 4.14.

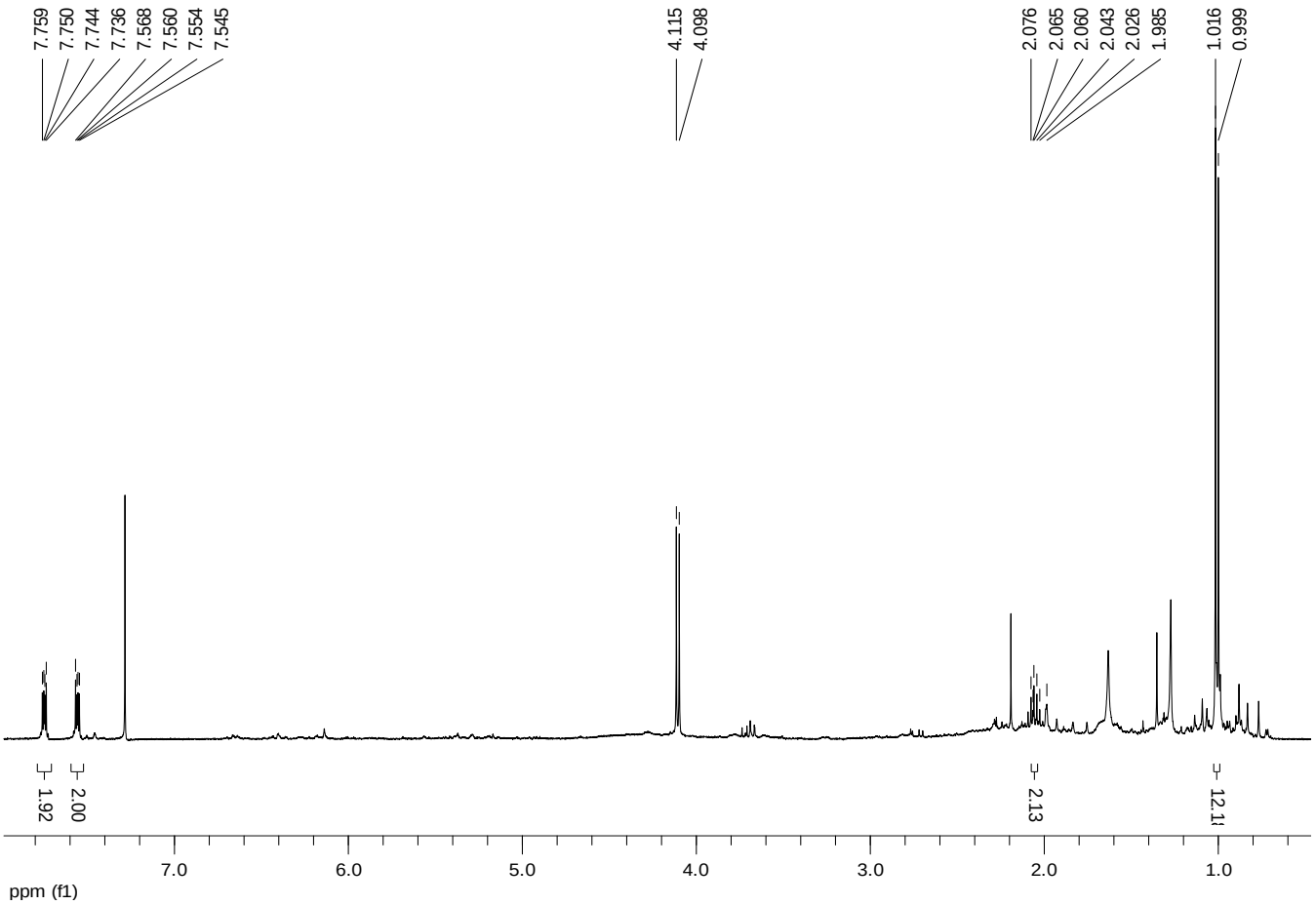


Figure 4.8. ^{1}H-NMR spectrum of BEE-28

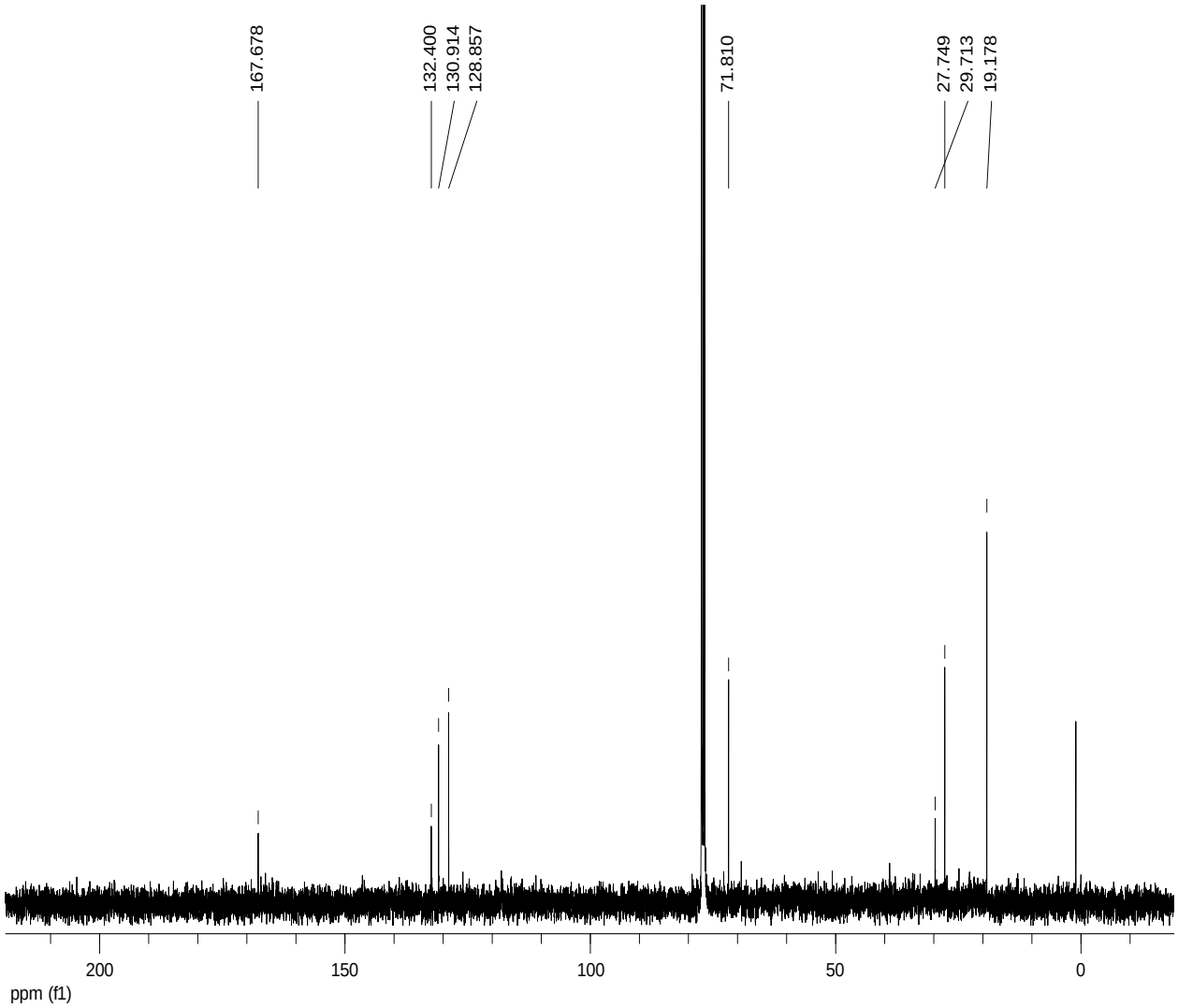


Figure 4.9. ^{13}C-NMR spectrum of BEE-28

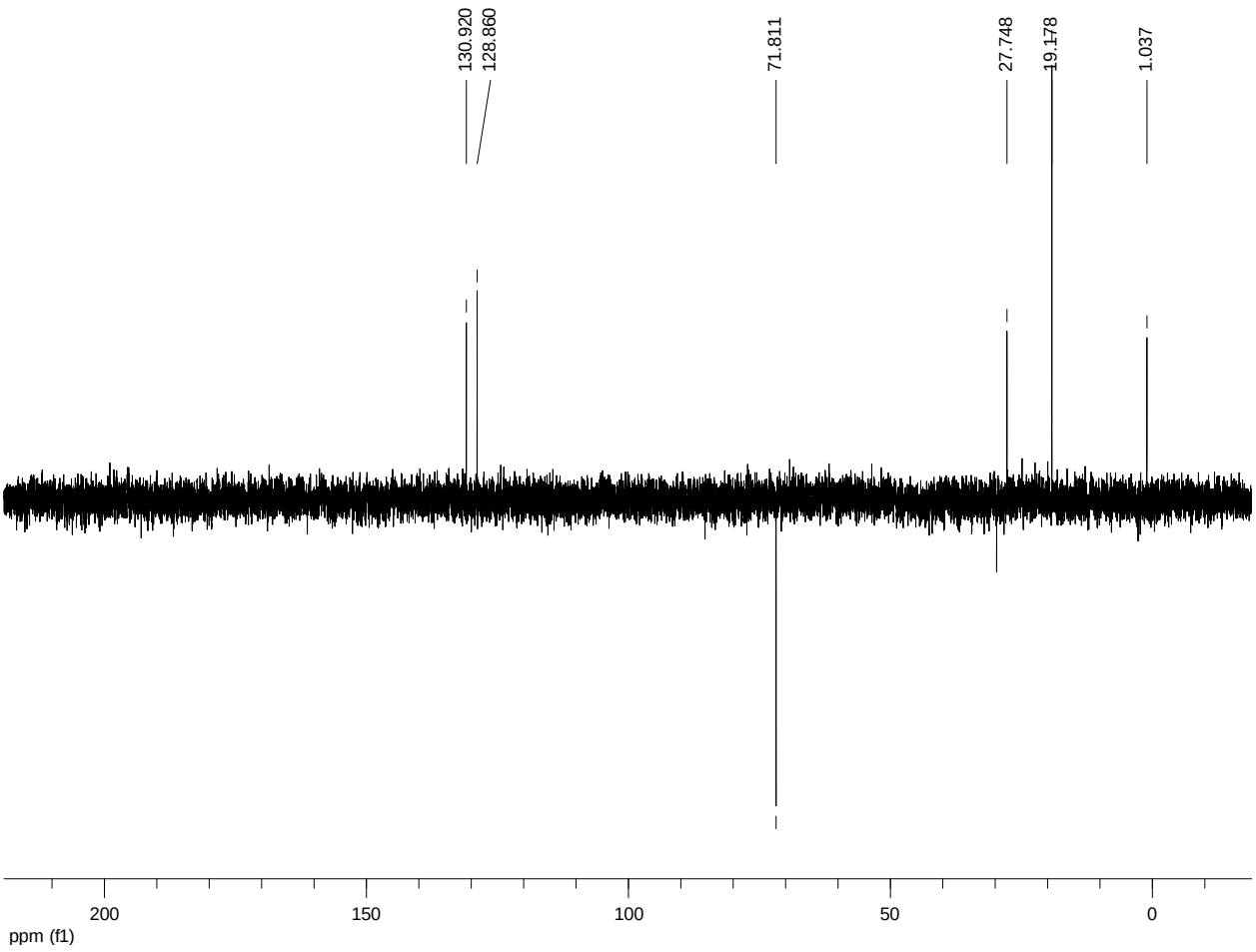
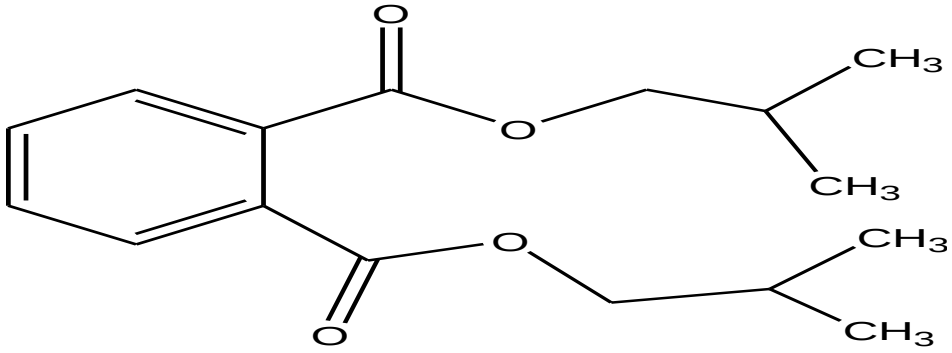


Figure 4.10. DEPT-135 NMR spectrum of BEE-28



Compound BEE-28

Figure 4. 11. Structure of compound BEE-28

4.9.2. Compound BEE-32

Compound BEE-32 was isolated as yellow needle like crystals melting point at 209-211ºC. The infra-red (IR) spectrum of compound BEE-32 showed several intense bands in the region of 4000 and 600 cm^{-1}. The band due to hydroxyl (O-H) stretching is evident at 3497 cm^{-1}. The IR spectrum also showed bands due to carbonyl group (C=O) at 1741 cm^{-1} and the band at 3000 cm-1 is accounted to C-H stretching of hydrocarbons (Figure 4.12).

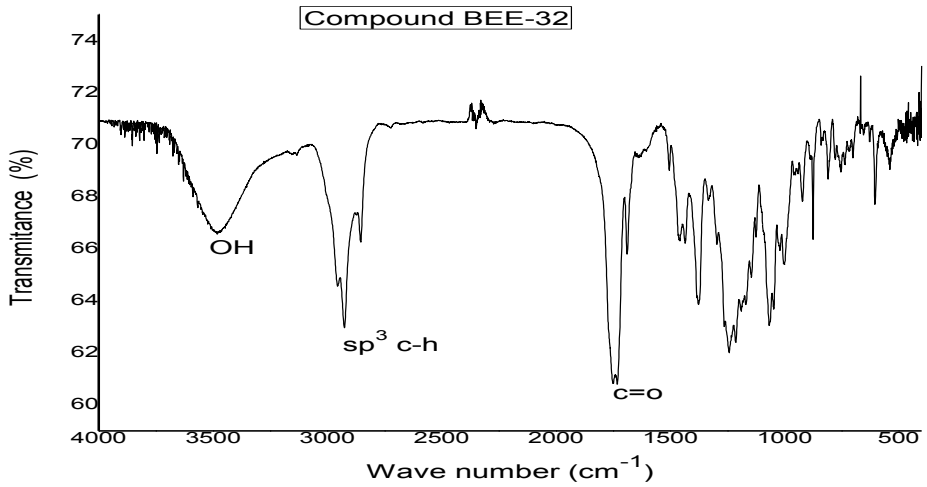


Figure 4.12. IR spectrum of compound BEE-32

The ^{1}H-NMR spectrum ($CDCl_3$) of compound BEE-32 showed signals at 7.52 (1H, s) and 7.40 (1H, s) accounted to the presence of hydrogen on furan ring. Another proton signal at 6.37 (1H) is accounted to the methine proton of the furan ring. The ^{1}H-NMR spectrum also showed signals in the oxygenated and non-oxygenated aliphatic carbon.

The ^{13}C-NMR spectrum of compound BEE-**32** with the help of DEPT-135 revealed the presence of 35 carbon resonances of which three methylene's, 13 methine, 8 methyl and the remaining 11 carbons are quaternary. The signal due to carbonyl compound of a ketone was observed at 204.5. The presence of five ester carbonyls are evident at 173.5, 170.7, 169.7, 168.6 and 166.6. Also observed in the aromatic region are signals due the furan ring at 143.0, 141.2, 120.3 and 109.9. The signals due to olefinic carbon were observed at 139.6 and 116.6. The spectrum also showed signals in the oxygenated and non-oxygenated aliphatic carbons.

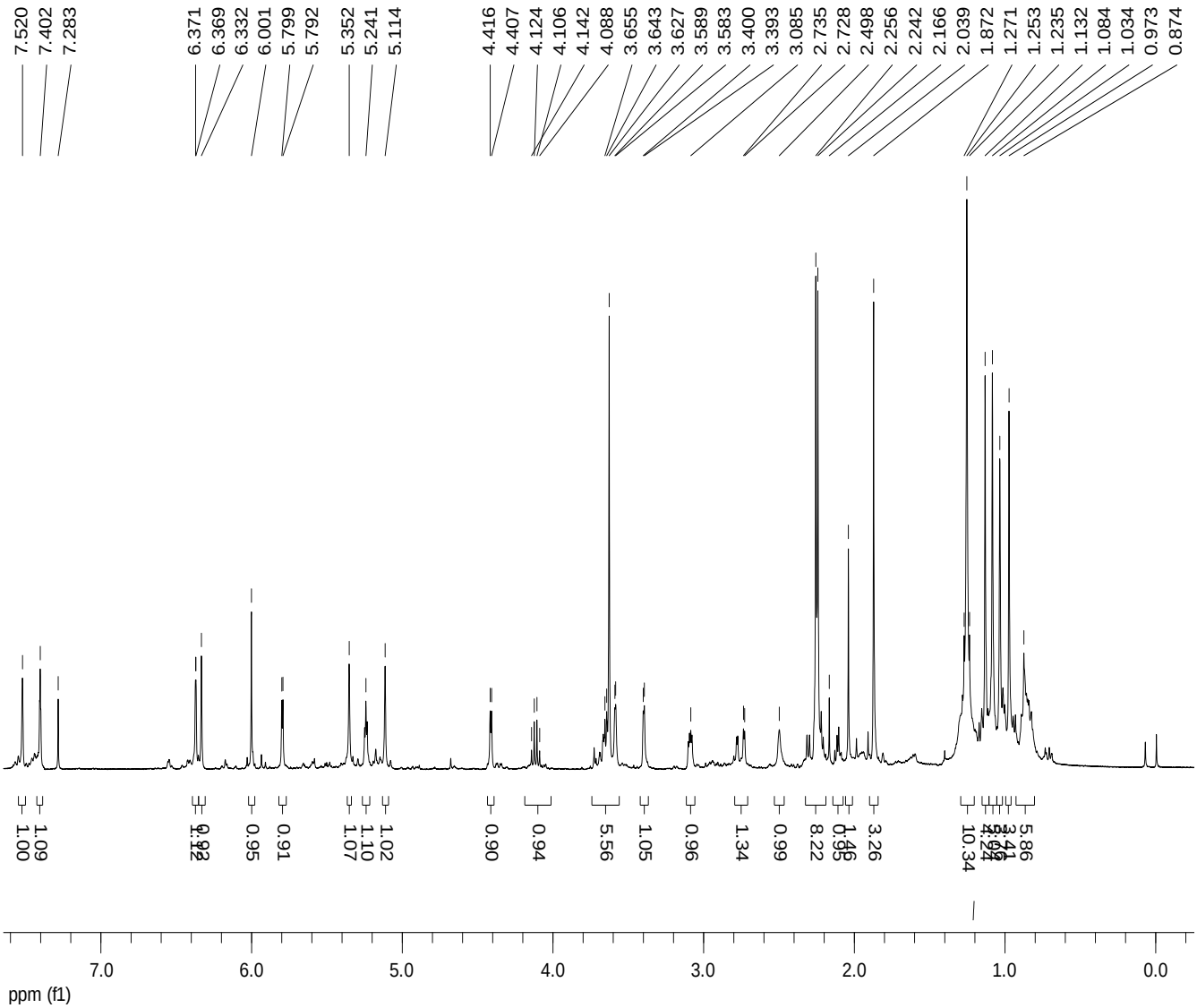


Figure. 4.13. ^{1}H-NMR spectrum of BEE-32

Table 4.6. ^{1}H-NMR spectral data of compound BEE 32

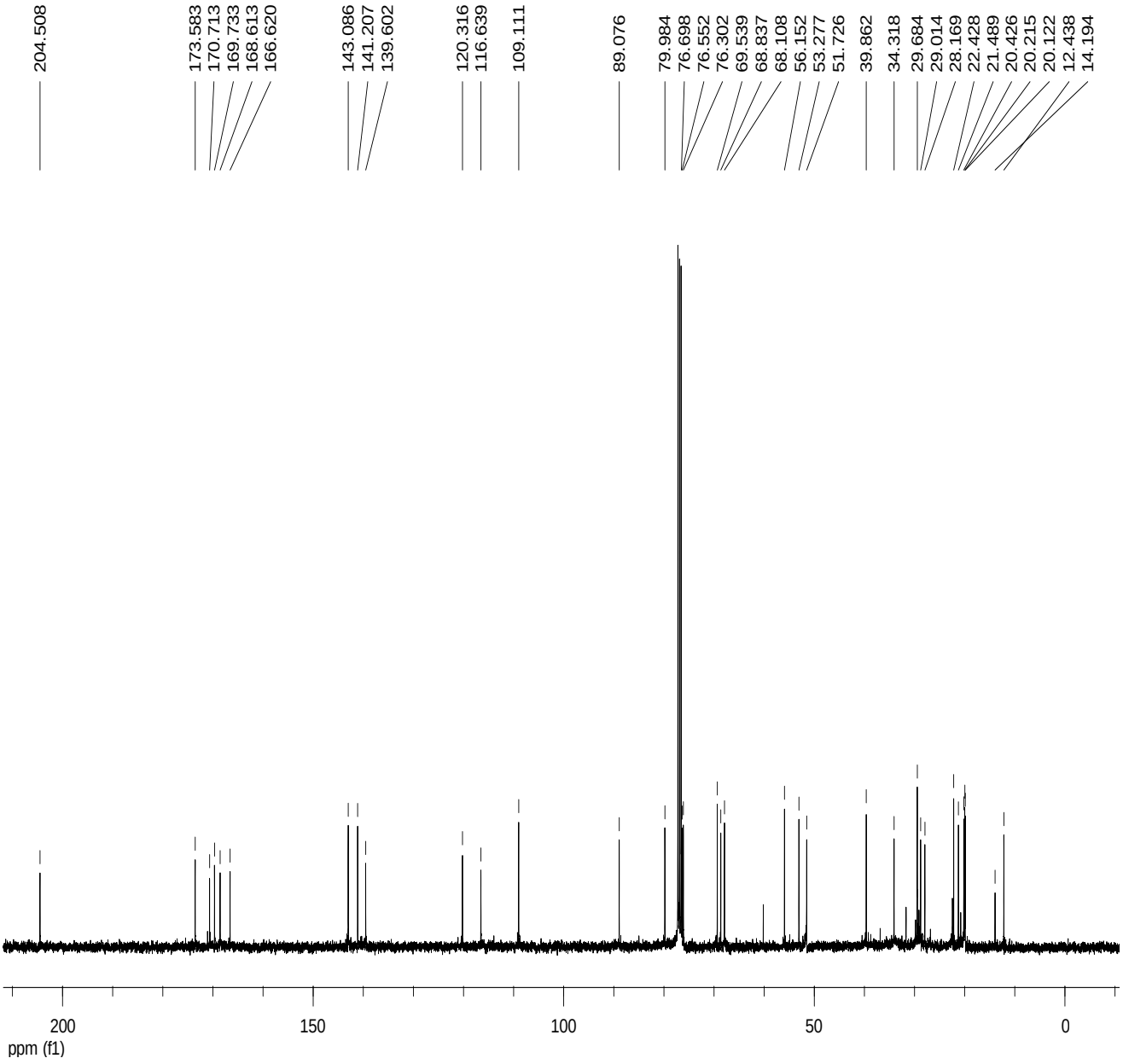


Figure 4. 14. ^{13}C-NMR spectrum of BEE-32

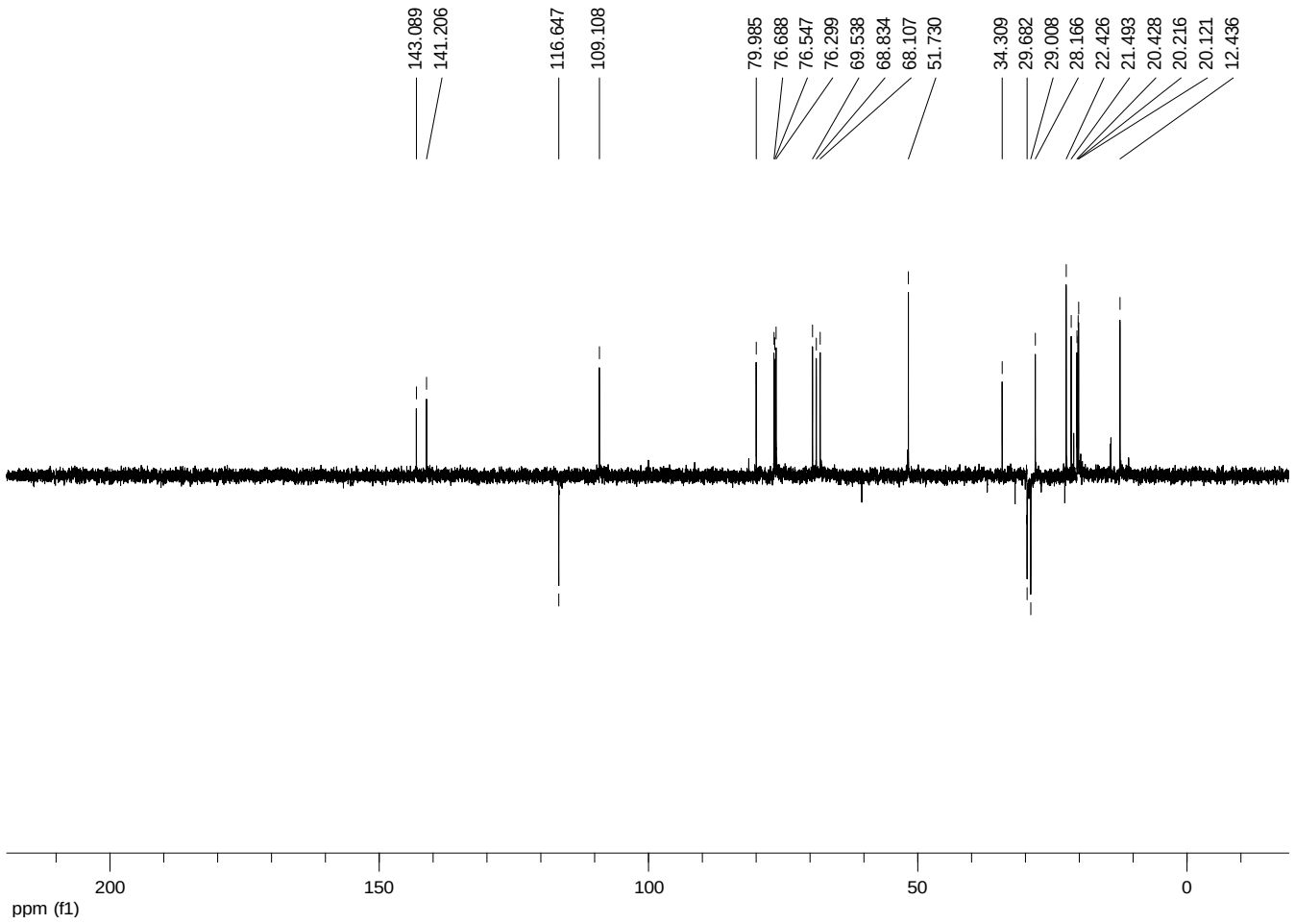
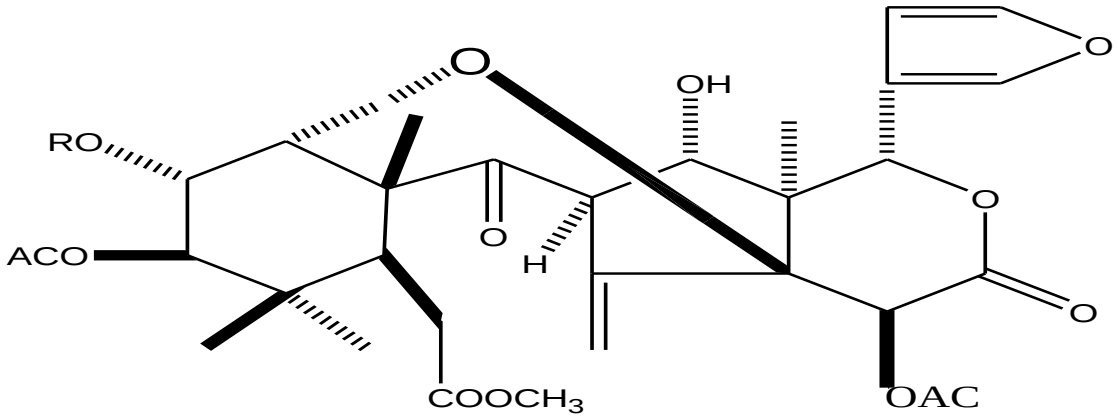


Figure 4.15. DEPT-135 NMR spectrum of BEE-32



Compound BEE-32

NB: RO=

Figure 4.16. Chemical structure of compound BEE-32

4.9.3. Compound BEE-29

The infra-red (IR) spectrum of compound BEE-29 showed several intense bands in the region of 4000 and 500 cm^{-1}. The IR spectrum (in KBr) indicated the presence of –OH group at 3500 cm^{-1}, C-H, conjugated carbonyl group and c=c between 2000 to1500 cm^{-1}, along with other absorption bands at 3500, 1400, 1000, and 500 cm^{-1}

The ^{1}H, ^{13}C and DEPT-135 NMR spectrum generated for characterization of compound BEE-28 indicated the presence of many peaks with the major one identical with the NMR data of compound 28.

5. DISCUSSION

The increase of microbial resistance to antibiotics created several problems. The severity of multidrug resistance issue is aggravated by the fact that there are no new antibiotics being discovered. The scientific and medical community should turn their efforts to find alternatives for antibiotics. One of the most common approaches is the search for the easiest and the safest to human, which are medicinal plants. Plant extracts do not pose selective pressure issues and unlikely to cause resistance problems.

One reason for choosing plants is that they are readily available. The entire world flora, 250,000 species have been identified and used for curative purposes (Patwardhan *et al*., 2005). This number represents only 15% of those species that have been effectively investigated and found useful (Okeke *et al*., 2005). Consequently, there is a staggering over 85% of higher plants, which are yet to be investigated.

During this study, two plants were collected from Oromia region North Shewa and one purchased from local markets of Adama. The extracts of these plants were obtained by soaking in hexane, ethyl acetate and methanol respectively. The extracts were obtained after filtration and evaporation. The highest percentage of yield extracted from each experimental plant was the ethyl acetate extracts of *E. capensis* which was 1.72% followed by Methanol extract of *Catha edulis* and *Premna resinosa* 1.62%, 1.01% respectively (Figure 4.1). This indicates that the secondary metabolites present in the barks of *E. capensis* are mainly medium polar. On the other hand, the leaves of *Catha edulis* and roots of *Premna resinosa* contain polar constituents.

Crude extracts of the plants may have mixtures of active compounds which act synergistically, and their overall bioactivity is usually greater than individual compounds (Tanver *et al.*, 2017). The percentage of extraction yields will increase or decrease with the ratio of solvent, temperature of extraction and extraction methods (green extractions, microwave assisted solvent extraction, ultrasound, etc) Tanver *et al*., 2017.

Bioactivity of plants extract significantly varied based on the solvents used for extraction, and depends on geographical source (Cervenka *et al*., 2006), harvest time, storage conditions, soil conditions, drying method, and rout extract (Bernard *et al*., 2014). The benefit of plant

properties against microorganisms can only be achieved by using a specific solvent and solvent concentration in extracting the plant materials.

In this study, ethyl acetate was selected as the extraction solvent because of the crude extract by ethyl acetate showed highest bioactivity against gram positive bacteria (*S. aureus* and laboratory isolates A and B and *C. albicans* and highest percentage of yield. Further, ethyl acetate extraction is widely used to obtain crude extracts of phytochemicals from plant. The concentration of the solvent could affect the antibacterial activity, it is necessary to select the appropriate solvent and its concentration.

Several studies are conducted to investigate the biological activities of plants (Bernard *et al*., 2014). Most of these studies tried to determine the antimicrobials activates of extracts obtained from plant materials. However, this study was to focus on to evaluate anti-QS and anti-biofilm activity of extracts and its purified compounds to explore the possibility of finding out new methods for prevention or treatment of infectious disease caused by biofilm forming microorganisms.

5.1. Antimicrobials Effect of Crude Extracts

This study indicated that considerable difference in antimicrobial activity between extracts obtained with different solvents. Ethyl acetate extracts were more active than other extracts against Gram- positive bacteria (*S. aureus*) and antifungals (*C. albicans*). No activity was observed by crude extract against Gram-negative bacteria (*P. aeruginosa*). The reason for the difference in activity between Gram positive and Gram- negative bacteria possibly lies in their morphological differences. The Gram-negative bacteria have an outer phospholipid membrane making their cell wall impermeable to lipophilic solutes. On the other hand, the Gram-positive bacteria lack this membrane and are thus more permeable (Nostro *et al*., 2000).

This study provided evidence that ethyl acetate extract of *E. capensis* possesses significant antibacterial (multi-drug resistance *S. aureus)* and antifungal activites on *C. albicans*. This activity may be due to the major components for *E. capensis* which include cinnamaldehyde and terpenoids (Budri *et al*., 2015). Similar results were obtained by Keskin and Toroglu (2011), and these results were also compatible with the result obtained by Utchariyakiat *et al*., (2016), where they showed a strong antibacterial activity of *C. zeylanicum* against *S. aureus*.

In addition, the results obtained in this study agreed with the result obtained by Krishnan and Nair (2016), which showed that the extract of *C. zeylanicum* had higher inhibitory effect against *S. aureus*. In relation to antifungal activity against *C. albicans*, a study by Castro and Lima (2013), showed MIC values ranging between 0.3 and 0.6 mg/ml. These results differ from the results of this study, which showed higher inhibitory effect at 10 mg/ml. A study by Alrumman (2015) showed that *T. aphylla* leaves possessed a significant antimicrobial activity against the human pathogens *S. aureus, Klebsiella pneumoniae, K. oxytoca, Proteus. mirabilis, P. aeruginosa, Micrococcus luteus* and *Shigella sp*., and *Candida sp*. This is in full agreement with our result against *S. aureus and C. albicans*.

Bioactivity of *T. aphylla* is greatly affected according to the method of extraction. Extractions from fresh materials have a more profound effect in terms of antimicrobial activities compared with dry ones. Dried plant materials are characterized by less volatile compounds that have been considered as being good antimicrobial agents (Jaber *et al*., 2012) The result obtained by Zarai *et al*., (2011), indicated the absence of inhibition zones and no planktonic growth inhibition activity (MIC) against *P. aeruginosa* for *M. vulgare* crude extract. This is like the case with this study, which also did not show any zone of inhibition. However, it showed activity when tested using purified compounds in the range of 5 and 10mg/ml

5.2. *In Vitro* Effect of Plant Crude Extracts on Biofilm Formation

The present study focused on the anti-biofilms and QS inhibitory effect of medicinal plant extracts. The activity of the extracts on inhibition of biofilm formation was tested using the crystal violet (CV) method, which is widely used by microbiologists. It is inexpensive and can be repeated many times to ensure accurate results (reproducible). The extracts used in this, showed antibacterial activity against *S. aureus* and *P. aeruginosa* at 10 and 5mg/ml concentration. The reason for the selection of *S. aureus* and *P. aeruginosa* in this assay is the fact that the biofilms forming ability of this bacterium contribute to its colonization in causing acute infections as well as its presence in burned tissue surrounding blood vessels and adipose cells (Schaber *et al*., 2007). Various results were obtained on inhibition of biofilm for *E. capensis, C. edulis* and *P. resinosa* extracts. *E. capensis* showed prominent antimicrobial and anti-biofilm activities. The result obtained by Latakian *et al*., (2016), agrees with our study. In this study, the *E. capensis* extracts tested prevented the formation of biofilm at concentration

of 10mg/mL, like a study by Pratiwi (2015), which prevented the formation of biofilm at concentration of 9mg/ml.

A low concentration of the extract may be required to prevent biofilm first attachment, while higher concentration to disrupt preformed biofilm (Stewart, 2002). In this study indicated that most plant extracts have the antibacterial coupled with anti-biofilm activity, therefore, may prove helpful for developing biofilm inhibitors and increase the effectiveness of infectious diseases treatment. In this study, *E. capensis* showed remarkable potential as anti-biofilm agent, as it was active upon *P. aeruginosa, S. aureus, C. albicans* in the planktonic state, no articles on anti-biofilm activity of extract of *E. capensis* was published, however it failed to reduce the QS in most assays.

The first discovered natural products inhibited QS and biofilm maturation in Gram-negative bacteria are the halogenated furanones from *Delisea pulchra*, and other QSI compounds such as cyclic sulphur derivatives obtained from garlic and patulin produced by *Penicillium* species (Persson *et al*., 2005). *E. capensis* extract showed strong anti-biofilm activity against *S. aureus* and *P. aeruginosa* compared to the untreated control. These medicinal plants could be used to manage *Pseudomonas* pathogenesis and hinder its dissemination. To the best of our knowledge, no reports are available regarding the anti-biofilm activity of *S. aureus P. aeruginosa* and *C. albicans* by *E. capensis* extract.

The anti-QS potential of plant extracts to explore the potential to a possible use in controlling detrimental pathogenic bacteria such as: *S. aureus P. aeruginosa*. This results in different system/pathway of quorum sensing by *S. aureus P. aeruginosa* and *C. albicans*. Therefore, there are different mechanisms of quorum sensing inhibition (Figure 2.3.) by active component from plant extract, either by inhibition the production of the virulence factor violacein, inhibition of two QS system effective compounds, and inhibition of QS system receptors (Loughlin *et al*., 2013).

We investigated if the extracts which inhibited *S. aureus*, *P. aeruginosa* and *C. albicans* motility as a mean for determining the inhibition of quorum sensing as an alternative strategy for controlling bacterial virulence as suggested by Vasavi *et al*., (2014). The swarming motility is important for cystic fibrosis acute infections, common cause of severe nosocomial infections, and involved at early stages of biofilm formation. Three major forms of motility are displayed by *S. aureus* and *P. aeruginosa* (swarming on semisolid on solid surfaces, and flagellum-mediated swimming in aqueous environments). The swarming assay depends on factors such as nutrient composition, agar type and composition, sterilization protocol (e.g., autoclaving), and semi-solid media curing among others, thus, it is highly sensitive to environmental factors. The edge of the colonies in smarming motility is highly irregular compared with control. Flagellar-mediated swimming motility is associated with biofilm formation by instigating the cell-to-surface attachment and plays an important role in the virulence of pathogens (Wu *et al*., 2014).

E. capensis extract reduced the swarming motility of *S. aureus* and *P. aeruginosa*. It might be because of phytochemicals of *E. capensis* on flagella-related processes, namely, flagella biosynthesis, rotation, rafting and chemotaxis, etc. Interestingly 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. In this study, we used molten soft top agar for all experiments about swarming motility. Now it is well established that most of these compounds have antimicrobial and even antifungal activity (Zhang *et al*., 2014). Interestingly, studies associated with some natural products have shown that plant extracts with poor antimicrobial properties could also be effective against bacterial biofilms (Upadhyay, 2014).

5.3. QS Inhibitory Activity of Plant Crude Extracts on Pasteurized Milk

In this study the untreated control and milk inoculated with *P. aeruginosa* exhibited spoilage, whereas, milk inoculated with the test organism and treated with crude extract showed minimal spoilage after 48h at room temperatures (Figure 4.6). Further, *in vitro* challenge studies at 10mg/ml concentrations prevented spoilage of milk by *P. aeruginosa*. It is well known that spoilage of milk by *Pseudomonas* species is due to their ability to produce the exoenzymes lipases and proteases (Dogan and Boor, 2003). Studies have shown that the exoenzyme production in gram-negative spoilage bacteria such as *Pseudomonas* and other *Enterobacteriaceae* members is under QS regulation.

5.4. Lethality Test of Extracts on the Leeches

Many plant species possess pharmacologically active constituents which contribute to their wide use in folk medicine and in the design of drugs. Due to the ethical issues in toxicological tests, substituting animals with alternative models is very important. The effectiveness of *E. capensis* bioassay for predicting the toxicity of plant extracts was evaluated by comparing the results for the brine shrimps at 2000 mg and 5000 mg (Obembe *et al*., 2014). In their study they used high concentration that four time of in this study. Another study on toxicity of *A. digitata* against Brine-shrimp of organic extract of *A. digitata* were non-toxic since both extracts had LC_{50} > 1000 µg/ml (Musila *et al*., 2013). In our study the cytotoxic effect of extract at 500mg of *E. capensis, C. edulis* and *P. resinosa* have been studied using leeches' lethality test. From the examined extract of these plants, the ethyl acetate extract *E. capensis* has shown positive result compared with the result obtained by (Obembe *et al*., 2014) and (Musila *et al*., 2013).

5.5. Characterization and Elucidation of Isolated Compounds

The air-dried bark of *E. capensis* were extracted separately with hexane, ethyl acetate and methanol at room temperature. The ethyl acetate extracts were subjected to column chromatography on silica gel because of yielding five secondary metabolites and bioactive, which characterized by NMR and IR. Out of the three constituents bioactive isolated from the back, one compound BEE-**32** similar structure with Sandrapin **B** but different by molecular formula and functional groups, whereas compound BEE **29** known as Diisobuylphyhalate

(Beatrice *et al*., 2014) and compound BEE **29** shows mixture absorbance in both ^{1}H-NMR and ^{13}C-NMR.

Compound BEE-**32** was obtained as yellow needles, like crystals melting point 209-211ºC. The infra-red (IR) spectrum of compound BEE-**32** showed several intense bands in the region of 4000 and 600 cm^{-1} and ^{1}H-NMR spectrum ($CDCl_3$) of compound BEE-32 showed signals at 7.52 (1H, s) and 7.40 (1H, s) accounted to the presence of hydrogen on furan ring. The proton signal at 6.37 (1H) and 6.36 (1H) are accounted to the methine proton of the furan ring. The ^{1}H-NMR spectrum also showed signals in the oxygenated and non-oxygenated aliphatic carbon.

The ^{13}C-NMR spectrum of compound BEE-**32** with the help of DEPT-135 revealed the presence of 35 carbon resonances of which three methylene's, 13 methine, 8 methyl and the remaining 11 carbons are quaternary. The signal due to carbonyl compound of a ketone was observed at 204.5. The presence of five ester carbonyls are evident at 173.5, 170.7, 169.7, 168.6 and 166.6. Also observed in the aromatic region are signals due the furan ring at 143.0, 141.2, 120.3 and 109.9. The signals due to olefinic carbon were observed at 139.6 and 116.6. Based on data from ^{1}H-NMR, ^{13}C-NMR and DEPT molecular formula of compound BEE-**32** were $C_{36}H_{46}O_{14}$. This compound isolated for the first time from bark of *E. capensis* and bioactivity of this compounds was not reported.

Compound BEE-**28** was isolated as purple colour, melting point at 77-78°C. The infra-red (IR) spectrum of compound BEE-28 showed several intense bands in the region of 4000 and 500 cm^{-1}. The ^{1}H-NMR spectrum of compound BEE-**28** have proton signals in the aliphatic and aromatic region. The doublet signal at 0.99 ppm integrating for twelve hydrogens is accounted to the presence of four methyl groups in the compound. The proton signal on oxygenated carbon is evident at 4.11 (d, 4H, J=7.6).

The ^{13}C-NMR spectrum of compound 28 with the aid of DEPT-135 have eight carbon resonances of which two quaternaries, three methane, two methyl and one methylene. The carbon signal at 167.6 ppm is accounted to the presence of carbonyl carbon of an ester. The three carbon signals in the aromatic region were evident at 132.4, 130.9 and 128.8 ppm. The signal due to an oxygenated methylene carbon was observed at 71.8 ppm.

The spectrum data obtained from 1H-NMR, 13-NMR and DEPT-135 NMR spectrum help to propose the structure and molecular formula ($C_{16}H_{22}O_4$) for compound BEE-**29** (Diisobutylphtalate). Diisobutylphtalate is an odourless plasticizer and has excellent heat and light stability. Diisobutylphtalate was reported from the stem of *Polyalthia rumphii* and showed anticancer activity on different cell lines (Wang *et al*., 2012). Even thought diisobutylphtalate was reported for first time from *E. capensis*

5. CONCULUSION AND RECOMENDATION

5.1. Conclusion

Biofilm and QS are major virulence factors of most of the pathogenic microorganism. Therefore, the finding of this present study was demonstrated that the potential of extracts and isolated bioactive compounds from *E. capensis* against antimicrobials, antibiofilm and anti-quorum sensing activity. These results may represent a call for examining the possibility of using these plants or their components in ethnomedicine and drug discovery from natural sources.

According to the findings of this study, the following conclusions were drawn:

- ✓ The ethyl acetate extract of *E. capensis* showed potential antimicrobials activity
- ✓ The highest antimicrobial activity against *C. albicans* and *S. aureus* was observed for *E. capensis*
- ✓ The results obtained in this study showed that ethyl acetate extracts of *E. capensis* inhibited biofilm formation against *S. aureus, P. aeruginosa*
- ✓ The extract of *E. capensis* constitute an interesting source for anti-biofilm agents in the development of new strategies to treat infections
- ✓ *E. capensis* extracts exhibited inhibition of the swarming motility of against *P. aeruginosa, S aureus C. albicans and* two laboratory isolates.
- ✓ *E*xtract of each of this experimental plant showed variable abilities in inhibiting quorum sensing through inhibiting the different virulence factors activity of *P. aeruginosa* in pasteurized and homogenised milk
- ✓ The study on toxicity of *E. capensis* against leeches confirmed that the extract has biological active
- ✓ Thin layer chromatography and Phytochemicals study of bark *E. capensis* shows five secondary metabolites including: alkaloids, flavonoids, saponin, terpenoids and tannin.
- ✓ Three bioactive compounds were isolated from the bark of *E. capensis* and characterized using IR, NMR and Melting point
- ✓ Two compounds (BEE-**29** $C_{16}H_{22}O_4$ and BEE-**32** C_{35} $H_{42}O_{14}$) were elucidated from bark *E. capensis* ethyl acetate extracts

5.2. Recommendation

Considering the above conclusions and based on the results of this study, the following recommendations are suggested:

- ✓ Further study should be conducted to synthesis an antimicrobial, antibiofilm and anti-quorum sensing agents of bioactive compounds from the bark of *E. capensis*.
- ✓ Further studies to confirm the findings of this study using other methods and experimenting on purified components instead of the crude extract to determine the biologically active component for each plant.
- ✓ Further investigations in animal models to determine plant extract toxicity (LD_{50}) and to human cells.
- ✓ It is recommended that plant extracts with bioactivities against biofilm and QS be further studied to check their efficacies.
- ✓ The compounds that have been characterized in this study exhibited a wide range of bioactivity on bacterial growth, but one compound didn't characterize. Since it has biological active so further studies needed to know the structure of this compound.
- ✓ There are very few reports available on the anti-biofilm and anti QS activities. Hence, we recommend further studies aiming at finding the anti-biofilm and anti QS activities of different plant extracts.

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Appendixes

Appendix A

Plant Vouchering Form

Collector: _____

Date Collected: _____

Family: _____

Scientific Name: _____

Verbal: _____

Locality: (State) _____

(District) _____

(Township) _____

Elevation: _____

Habitat: _____

Occurrence: (Common) ___ (Occasional)____ (Rare) ____

Plant Height: _____

Flower Colour: _____

Seed: _____

Fruit: _____

Edible / Non-edible: _____

Medicinal Plant (Usage)_____

Appendix B

Table 5.1. Masses of extract obtained and yields in %

Plant species	Part used	Solvents extract	Amount extracted (g)	% yield
Catha edulis	Leaves	Hexane	2.78	1.13%
		Ethyl acetate	3.6	1.47%
		Methanol	3.98	1.62%
Ekebergia capensis	Barks	Hexane	3.2	1.17%
		Ethyl acetate	4.7	1.72%
		Methanol	3.6	1.32%
Premna resinosa	Roots	Hexane	2.1	0.17%
		Ethyl acetate	2.40	0.90%
		Methanol	2.76	1.01%