# DETECTION OF BIOACTIVE CONSTITUENTS IN *PHYLLANTHUS AMARUS* USING THIN LAYER CHROMATOGRAPHY-BIOAUTOGRAPHIC METHOD AGAINST *STAPHYLOCOCCUS AUREUS* AND *COAGULASE NEGATIVE STAPHYLOCOCCUS AUREUS*


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## CERTIFICATION PAGE

We certify that this project work was carried out by ORIRE, OMOLOLA VICTORIA with the Matriculation Number MCB/11/0346 of the Department of Microbiology, Faculty of Science, Federal University Oye Ekiti, under the supervision of Dr S.K. OJO

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## DEDICATION

This project work is dedicated to God, for his help towards the success of this work.

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## ABSTRACT

Medicinal plants have been used for centuries as remedies for human diseases because they contain components of therapeutic value. Phyllanthus amarus has shown to work as an antifungal, antibacterial and antiviral agent. Bioautography is a technique used to isolate compounds by employing a suitable chromatographic process followed by a biological detection system. Referenced pure culture antibiotics resistant Coagulase Negative Staphylococcus strain (CoNS 10b: Ojo) and Staphylococcus aureus (W241: Ojo) were obtained from the Drug Discovery and Development Research (DDDR) unit, Department of Microbiology, Federal University Oye Ekiti, confirmation tests were carried out on these organisms which showed that the two strains were Gram positive, cocci and ferment various sugars with production of acid. The extract of Phyllanthus amarus was gotten through both cold and hot (soxhlet) extraction, Thin Layer Chromatography-Bioautographic method was carried out on the extracts to detect the antibacterial activity of various bioactive constituent in Phyllanthus amarus against Staphylococcus strains. The result revealed various retention factors of the extracts in various solvent systems ranging from 0.096-0.838, with the total number of active bands ranging from 1-7. Different zones of inhibition were also recorded, ranging from 18mm-27mm. The result shows that there are bioactive constituents present in Phyllanthus amarus and Bioautography (agar-overlay) can be used to detect them.

# CHAPTER ONE

## 1.0 INTRODUCTION

Throughout the history of drug development, natural products have provided a fundamental source of drugs for fighting infections. Like in many developing countries, new drugs are often not affordable in Nigeria. Approximately 60–80% of the world's population still relies on traditional medicines as remedies for the treatment of common illnesses (Owolabi *et al.*, 2007). Nascimento *et al.,* (2000) stated that the World Health Organization (W.H.O) has ascribed medicinal plants as the best source of variety of drugs in combating serious diseases and it advocates that countries should venture into other aspects of traditional medicine. This should be with a view of identifying safe and effective remedies for ailments of both microbial and non-microbial organisms.

Medicinal plants have been used for centuries as remedies for human diseases because they contain components of therapeutic value (Tanaka *et al.*, 2002). In recent years, secondary plant metabolites (phytochemicals) have been extensively investigated as a source of medicinal agents. It is anticipated that phytochemicals with good antimicrobial activity will be used for the treatment of bacterial infections, fungi, and viruses. (Odetola and Akkoyenu, 2000)

The *Phyllanthus* genus belonging to the Euphorbiaceae family was first identified in Central and Southern India in 18th century but is now found in many countries including Philippines, China, Cuba and Nigeria among others. (Adeneye *et al.*, 2006). The Phyllanthus genus contains over 600 species of shrubs, trees and annual or biennial herbs distributed throughout the tropical and subtropical regions of both hemispheres (Taylor, 2003). *P amarus* is locally called Eyin olobe (Yoruba, south-west Nigeria), English (stone breaker or kidney stone

plant), Igbo (ngwu) (Adeneye *et al.*, 2006). In a number of countries, the aerial part of *Phyllanthus amarus* is highly valued in traditional medicine for its healing properties. This plant is traditionally used around the world in the treatment of liver ailments and kidney stones. *Phyllanthus amarus* has also shown to work as an antifungal, antibacterial and antiviral agent (Kassuy *et al.*, 2003). Chevallier (2000) pointed out that *P. amarus* is also used traditionally in India to treat cardiovascular problems.

Bioautography is a means of target-directed isolation of active molecules on chromatogram. They offer a rapid and easy identification of bioactive lead in complex matrices of plant extracts. Bioautography is a technique used to isolate compounds by employing a suitable chromatographic process followed by a biological detection system (Muller *et al.*, 2004). It is an effective and inexpensive technique for the phytochemical analysis of plant extracts to identify bioactive compounds. It can thus be performed both in highly developed laboratories as well as in small research laboratories which have minimum access to sophisticated equipments (Marston *et al.*, 2015).

**RESEARCH PROBLEM (TLC)**

Inadequate assay and pious knowledge has limited the use of various natures' flora (medicinal plants) that is abundant in our environment in Orthodox (Western) medicine practice.

**AIM OF STUDY**

Detection of bioactive constituents presents in *Phyllanthus amarus* using Bioautographic (Agar-overlay) method of the Thin Layer Chromatography against *S.aureus* and Coagulase negative *Staphylococcus*.

## OBJECTIVES OF STUDY

1. To perform a confirmatory test on the referenced strain using culturing and biochemical methods.

2. To carry out hot (soxhlet) and cold crude extraction method in different solvents.

3. To determine the various active bands in *Phyllanthus amarus* using various solvent system or concentrations.

4. To detect the antimicrobial activities of various active bands of different solvents against *S.aureus* and Coagulase Negative *Staphylococcus* strains.

# CHAPTER TWO

## 2.0 LITERATURE REVIEW

### 2.1 Medicinal plants

Plants have been used by man to cure diseases and heal injuries since ancient times. The universal role of plants in the treatment of diseases is illustrated by their employment in all major systems of medicine irrespective of the underlying philosophical premise. Plants have been used throughout the world in folk medicines as a local cure for common ailments (Ikekwe *et al.*, 2000). Plants are enriched with various natural products or phytochemical molecules such as lignins, Phyllanthin, hypophyllanthin, alkaloids, tannins, acids, quinines. The history of natural product in plants use in ancient times and in folk medicine around the world is the basis for the use of many therapeutic drugs in modern-day medicine. Traditionally, natural plant products have been the source in the search for new drugs by pharmaceutical companies (Adeneye *et al.*, 2006).

There has been an increasing interest worldwide on therapeutic values of natural products. It is believed that the cure of any debilitating human ailments and diseases may be found among the world's flora in natures pharmacy (Olowosulu and Ibrahim, 2006) and there are multitudes of potential useful bioactive substances to be derived from these plants. These Phytochemicals have made significant contribution in maintaining human health. The significant of drugs derived from plants cannot be over emphasized with the recent trend of high percentage of resistance of microorganisms to the present day antibiotics (Ikekwe *et al.*, 2000). This popular medicinal herb is also a remedy around the world for influenza, dropsy, diabetes and jaundice. Medicinal plants, vegetables and fruits are the sources of huge number of bioactive lead with therapeutic and nutraceutical importance.

## 2.2 Description of *Phyllanthus amarus*

The plant, *Phyllanthus amarus*, is a glabrous shrub or woody climber, found in savannah and drier secondary forests common in coastal thickets and scrub and widespread in parts of tropical Africa. *P.amarus* is an erect annual herb of not more than one and half feet tall and has small leaves and yellow flowers. It has been used traditionally in the treatment of various ailments (Adeneye *et al.*, 2006).

A number of the *Phyllanthus* species have been reported to have extensive history in medicine systems. Substantial amount of secondary metabolites present in the genus are used widely in traditional medicine for the treatment of flu, dropsy, diabetes, jaundice, gall bladder disease and liver disease (Kassuy *et al.*, 2003). It is an herb that grows up to 10-60cms tall and erect. It is mostly used by traditional healers. *P. amarus*, are used in the treatment for kidney and gallstones, other kidney related problems, appendix inflammation, and prostate problems. In many countries around the world plants in the genus *Phyllanthus* are used in folk remedies; therefore this genus is of great importance in traditional medicine. The genus *Phyllanthus* has a long history of use in the treatment of liver, kidney and bladder problem, diabetes and intestinal parasites. In folk medicine *P. amarus* has reportedly been used to treat jaundice, diabetes, otitis, diarrhoea, swelling, skin ulcer, gastrointestinal disturbances, and weakness of male organ and blocks DNA polymerase in the case of hepatitis B virus during reproduction (Oluwafemi and Debiri, 2008).

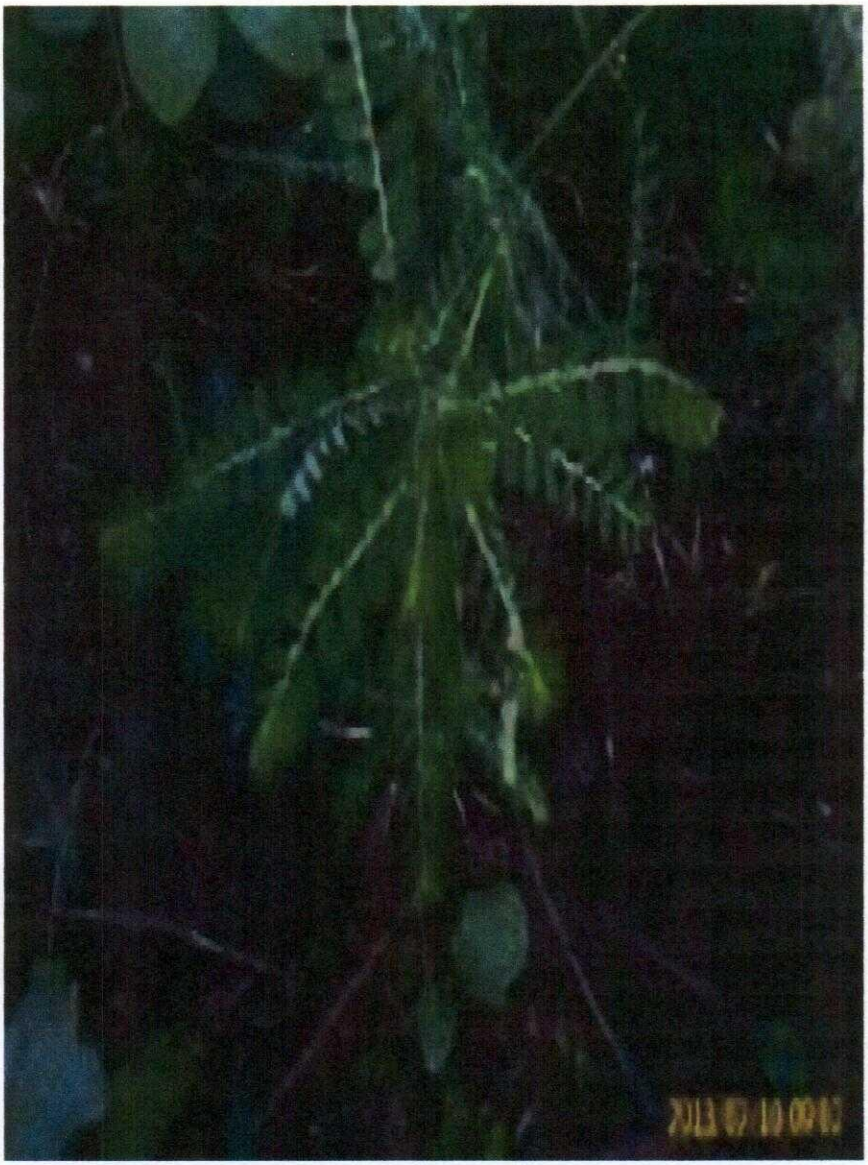

**Plate 1. Photograph of *Phyllanthus amarus* plant.**

## 2.3 Description of *Staphylococcus aureus*

### 2.3.1 Morphology

*Staphylococcus aureus* is a gram-positive coccal bacterium that is a member of the Firmicutes, and is frequently found in the respiratory tract and on the skin where perspiration is present. It is often positive for catalase and nitrate reduction (Ogston, 2000)

*S. aureus* (Greek "grape-cluster berry", Latin aureus, "golden") is a facultative anaerobic, gram-positive coccal bacterium also known as "golden staph" and Oro staphira. In medical literature, the bacterium is often referred to as *S. aureus* or *Staph aureus*. *S. aureus* appears as grape-like clusters when viewed through a microscope, and has large, round, golden-yellow colonies, often with hemolysis, when grown on blood agar plates (Ryan *et al.*, 2004) *S. aureus* reproduces asexually by binary fission; the two daughter cells do not fully separate and remain attached to one another, so the cells are observed in clusters. *S. aureus* is catalase-positive (meaning it can produce the enzyme catalase). Catalase converts hydrogen peroxide ($H_2O_2$) to water and oxygen. Catalase-activity tests are sometimes used to distinguish staphylococci from enterococci and streptococci.

### 2.3.2 Pathogenesis

*Staphylococcus aureus* has long been recognized as an important pathogen in human disease. Due to an increasing number of infections caused by methicillin-resistant *S. aureus* (MRSA) strains, therapy has become problematic. The ecological niches of *S. aureus* are the anterior nares (Klutymans, 2015). Various virulence factors contribute to the ability of *S. aureus* to cause infection which includes: enzymes, toxins, adhesion proteins, cell-surface proteins.

### 2.3.3 Transmission

*S. aureus* is most often spread to others by contaminated hands. The skin and mucous membranes is usually an effective barrier against infection. However, if these barriers are breached (e.g., skin damage due to trauma or mucosal damage due to viral infection*)* *S. aureus* may gain access to underlying tissues or the bloodstream and cause infection. Persons who are immunocompromised or who have invasive medical devices are particularly vulnerable to infection (Klutymans, 2015).

### 2.2.4 CLINICAL DIAGNOSIS

#### 2.2.4.0 Rapid diagnosis and typing

Diagnostic microbiology laboratories and reference laboratories are keys for identifying outbreaks and new strains of *S. aureus*. Recent genetic advances have enabled reliable and rapid techniques for the identification and characterization of clinical isolates of *S. aureus* in real time. These tools support infection control strategies to limit bacterial spread and ensure the appropriate use of antibiotics. Quantitative PCR is increasingly being used to identify outbreaks of infection (Mackay, 2007 and Francois, 2008).

When observing the evolvement of *S.aureus* and its ability to adapt to each modified antibiotic, two basic methods known as "band-based" or "sequence-based" are employed. Other methods such as multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), bacteriophage typing, spa locus typing, and SCCmec typing are often conducted more than others. With these methods, it can be determined where strains of MRSA originated and also where they are currently.

### 2.3.5 Treatment and antibiotic resistance

The treatment of choice for *S. aureus* infection is penicillin. An antibiotic derived from *Penicillum* fungus, penicillin inhibits the formation of peptidoglycan cross-linkages that provide the rigidity and strength in a bacterial cell wall. The four-membered β-lactam ring of penicillin is bound to enzyme DD-transpeptidase, an enzyme that when functional, cross-links chains of peptidoglycan that form bacterial cell walls (Korzeniowski, 2001). The binding of β-lactam to DD-transpeptidase inhibits the enzyme's functionality and it can no longer catalyze the formation of the cross-links. As a result, cell wall formation and degradation are imbalanced, thus resulting in cell death. In most countries, however, penicillin resistance is extremely common, and first-line therapy is most commonly a penicillinase-resistant β-lactam antibiotic (for example, oxacillin or flucloxacillin, both of which have the same mechanism of action as penicillin). Combination therapy with gentamicin may be used to treat serious infections, such asendocarditis (Chambers, 2001). But its use is controversial because of the high risk of damage to the kidneys (Cosgrove, 2009).The duration of treatment depends on the site of infection and on severity. Antibiotic resistance in *S. aureus* was uncommon when penicillin was first introduced in 1943. Indeed, the original Petri dish on which Alexander Fleming of Imperial College London observed the antibacterial activity of the *Penicillium* fungus was growing a culture of *S. aureus*. By 1950, 40% of hospital *S. aureus* isolates were penicillin-resistant; by 1960, this had risen to 80% (Chamber, 2001).

MRSA and often pronounced /ˈmɜrsə/ or /ɛm ɑ: ɛs eɪ/, is one of a number of greatly feared strains of *S. aureus* which have become resistant to most β-lactam antibiotics. For this reason, vancomycin, a glycopeptide antibiotic, is commonly used to combat MRSA. Vancomycin inhibits the synthesis of peptidoglycan, but unlike β-lactam antibiotics, glycopeptide antibiotics target and bind to amino acids in the cell wall, preventing peptidoglycan cross-

linkages from forming. MRSA strains are most often found associated with institutions such as hospitals, but are becoming increasingly prevalent in community-acquired infections. A recent study by the Translational Genomics Research Institute showed that nearly half (47%) of the meat and poultry in U.S. grocery stores were contaminated with *S. aureus*, with more than half (52%) of those bacteria resistant to antibiotics. This resistance is commonly caused by the widespread use of antibiotics in the husbandry of livestock, including prevention or treatment of an infection, as well as promoting growth.

Researchers from Italy have identified a bacteriophage active against *S. aureus*, including MRSA, in mice and possibly humans (Capparelli, 2007)

## 2.4 Antibiotics resistance in *Staphylococcus aureus*

### 2.4.1 Mechanism of antibiotic resistance

Staphylococcal resistance to penicillin is mediated by penicillinase (a form of β-lactamase) production: an enzyme that cleaves the β-lactam ring of the penicillin molecule, rendering the antibiotic ineffective. Penicillinase-resistant β-lactam antibiotics, such as methicillin, nafcillin, oxacillin, cloxacillin, dicloxacillin, andflucloxacillin, are able to resist degradation by staphylococcal penicillinase.

Resistance to methicillin is mediated via the *mec* operon, part of the staphylococcal cassette chromosome mec (SCC*mec*). Resistance is conferred by the *mecA* gene, which codes for an altered penicillin-binding protein (PBP2a or PBP2') that has a lower affinity for binding β-lactams (penicillins, cephalosporins, and carbapenems). This allows for resistance to all β-lactam antibiotics, and obviates their clinical use during MRSA infections. As such, the glycopeptide vancomycin is often deployed against MRSA.

Aminoglycoside antibiotics, such as kanamycin, gentamicin, streptomycin, etc., were once effective against staphylococcal infections until strains evolved mechanisms to inhibit the aminoglycosides' action, which occurs via protonated amine and/or hydroxyl interactions with the ribosomal RNA of the bacterial 30S ribosomal subunit. There are three main mechanisms of aminoglycoside resistance mechanisms which are currently and widely accepted: aminoglycoside modifying enzymes, ribosomal mutations, and active efflux of the drug out of the bacteria. Aminoglycoside-modifying enzymes inactivate the aminoglycoside by covalently attaching either a phosphate, nucleotide, or acetylmoiety to either the amine or the alcohol key functional group (or both groups) of the antibiotic. This changes the charge or sterically hinders the antibiotic, decreasing its ribosomal binding affinity. In *S. aureus*, the best-characterized aminoglycoside-modifying enzyme is aminoglycoside adenylyltransferase 4' IA (*ANT(4')IA*). This enzyme has been solved by x-ray crystallography. The enzyme is able to attach an adenyl moiety to the 4' hydroxyl group of many aminoglycosides, including kamamycin and gentamicin. Glycopeptide resistance is mediated by acquisition of the *vanA* gene, which originates from the enterococci and codes for an enzyme that produces an alternative peptidoglycan to which vancomycin will not bind. Today, *S. aureus* has become resistant to many commonly used antibiotics. In the UK, only 2% of all *S. aureus* isolates are sensitive to penicillin, with a similar picture in the rest of the world. The β-lactamase-resistant penicillins (methicillin, oxacillin, cloxacillin, and flucloxacillin) were developed to treat penicillin-resistant *S. aureus*, and are still used as first-line treatment. Methicillin was the first antibiotic in this class to be used (it was introduced in 1959), but, only two years later, the first case of MRSA was reported in England (Johnson, 2001).

Despite this, MRSA generally remained an uncommon finding, even in hospital settings, until the 1990s, when the MRSA prevalence in hospitals exploded and it is now endemic.

MRSA infections in both the hospital and community setting are commonly treated with non-β-lactam antibiotics, such asclindamycin (a lincosamine) and co-trimoxazole (also commonly known as trimethoprim/sulfamethoxazole). Resistance to these antibiotics has also led to the use of new, broad-spectrum anti-gram-positive antibiotics, such as linezolid, because of its availability as an oral drug. First-line treatment for serious invasive infections due to MRSA is currently glycopeptide antibiotics (vancomycin and teicoplanin). A number of problems with these antibiotics occur, such as the need for intravenous administration (no oral preparation is available), toxicity, and the need to monitor drug levels regularly by blood tests. Also, glycopeptide antibiotics do not penetrate very well into infected tissues (this is a particular concern with infections of the brain and meningesand in endocarditis). Glycopeptides must not be used to treat methicillin-sensitive *S. aureus* (MSSA), as outcomes are inferior (Blot, 2002) Because of the high level of resistance to penicillins and because of the potential for MRSA to develop resistance to vancomycin, the U.S. Centers for Disease Control and Prevention has published guidelines for the appropriate use of vancomycin. In situations where the incidence of MRSA infections is known to be high, the attending physician may choose to use a glycopeptideantibiotic until the identity of the infecting organism is known. After the infection is confirmed to be due to a methicillin-susceptible strain of *S. aureus*, treatment can be changed to flucloxacillin or even penicillin, as appropriate.

Vancomycin-resistant *S. aureus* (VRSA) is a strain of *S. aureus* that has become resistant to the glycopeptides. The first case of vancomycin-intermediate *S. aureus* (VISA) was reported in Japan in 1996; but the first case of *S. aureus* truly resistant to glycopeptide antibiotics was only reported in 2002 (Chang, 2003). Three cases of VRSA infection had been reported in the United States as of 2005 (Menichetti, 2005).

## 2.5 PREVENTION AND CONTROL OF INFECTION

### 2.5.1 Proper hand-wash and use of disposable gloves

Spread of *S. aureus* (including MRSA) generally is through human-to-human contact, although recently some veterinarians have discovered the infection can be spread through pets with environmental contamination thought to play a relatively unimportant part. Emphasis on basic hand washing techniques is, therefore, effective in preventing its transmission. The use of disposable aprons and gloves by staff reduces skin-to-skin contact and, therefore, further reduces the risk of transmission (Sing, 2008).

Recently, myriad cases of *S. aureus* have been reported in hospitals across America. Transmission of the pathogen is facilitated in medical settings where healthcare worker hygiene is insufficient. *S. aureus* is an incredibly hardy bacterium, as was shown in a study where it survived on polyester for just less than three months; polyester is the main material used in hospital privacy curtains (Neely, 2000). The bacteria are transported on the hands of healthcare workers, who may pick them up from a seemingly healthy patient carrying a benign or commensal strain of *S. aureus*, and then pass it on to the next patient being treated. Introduction of the bacteria into the bloodstream can lead to various complications, including endocarditis, meningitis, and, if it is widespread, sepsis.

### 2.5.2 Use of ethanol as sanitizer

Ethanol has proven to be an effective topical sanitizer against MRSA. Quaternary ammonium can be used in conjunction with ethanol to increase the duration of the sanitizing action. The prevention of nosocomial infections involves routine and terminal cleaning. Nonflammable alcohol vapor in CO 2 NAV-CO2 systems have an advantage, as they do not attack metals or plastics used in medical environments, and do not contribute to antibacterial resistance (Neely, 2000).

### 2.5.3 Use of antibiotics containing materials

An important and previously unrecognized means of community-associated MRSA colonization and transmission is during sexual contact. Staff or patients who are found to carry resistant strains of *S. aureus* may be required to undergo "eradication therapy", which may include antiseptic washes and shampoos (such as chlorhexidine) and application of topical antibiotic ointments (such as mupirocin or neomycin) to the anterior nares of the nose. *S. aureus* is killed in one minute at 78°C and in ten minutes at 64°C.

### 2.5.4 Biological control

Biological control might be a new possible way to control S. aureus in body surfaces. Colonization of body surfaces (especially in the nose) by S. epidermidis (inhibitory strain JK16) impairs the establishment of *S. aureus*.

According to Shafiei (2011), a study made points to this new possible way to control *S. aureus*. This study was performed from observations of the nasal microbial flora of a diverse group of people. Two different strains of S. epidermidis occur, one that inhibits biofilm formation by *S. aureus*, *S. epidermidis* strain JK16 (inhibitory type), and one that does not (non-inhibitory type) *S. epidermidis* strain JK11. Some patients were not affected by *S. aureus,* because these patients had S. aureus together with *S. epidermis* (inhibitory type), in their nasal microbial flora. This is due to an amensalistic relationship between these microorganisms, the inhibitory strain of *S. epidermidis* and *S. aureus*. These findings open the way to a biological control therapy to help in the treatment of S. aureus infections which are becoming a growing threat due to the rise of resistance to conventional antibiotic treatments.

## 2.6 BIOAUTOGRAPHY

Organic solvents employed in chromatographic separation process can be completely removed before biological detection because these solvents cause inactivation of enzymes and/or death of living organisms. Bioautography is a technique to isolate compounds by employing a suitable chromatographic process followed by a biological detection system. Planar chromatographic analysis hyphenated with the biological detection method is termed as bioautography (Muller *et al.,* 2004). Despite having sophisticated on-line high-performance liquid chromatography coupled bioassays, bioautography offers a simple, rapid and inexpensive method for the chemical and biological screening of complex plant extracts, with subsequent bioassay-guided isolation (Hostettmann *et al.*, 2015). According to Saikat *et al.*, (2015). Goodall and Levi in 1946 introduced paper chromatography (PC)-based bioautography for the first time to estimate the purity of penicillin. In 1961, Fisher and coworkers introduced thin layer chromatography (TLC)-based bioautography. The first review on bioautography was written by Betina in 1973. The major applications of bioautography are the fast screening of a large number of samples for bioactivity, namely, antibacterial, antifungal, antioxidant, enzyme inhibition, etc. and in the target-directed isolation of active compounds (Houghton, 2000). In this review, the techniques and application of bioautography are discussed in details with suitable examples.

### 2.6.1 Detection of Anti microbial agents by Bioautography

In contact bioautography, antimicrobial agents diffuse from a developed TLC plate or paper to an inoculated agar plate. The chromatogram is placed face down onto the inoculated agar layer for a specific period to enable diffusion. Then the chromatogram is removed and the agar layer is incubated. The zones of inhibition on the agar surface, corresponding to the spots in chromatographic plates, are indicative of the antimicrobial substances. (Sherman,

2008). Incubation time for the growth ranges between 16 and 24 h but it can be reduced to 5–6 h by spraying with 2,6-dichlorophenol-indophenol or 2,3,5- tetrazolium chloride (Shahat *et al.,* 2008) . The disadvantages of contact bioautography according to Saikat *et al.,* (2015) are:

- Difficulties in obtaining complete contact between the agar and the plate and adherence of the adsorbent to the agar surface.
- Another problem may arise due to the differential diffusion of components, especially water-insoluble, from the chromatogram to the agar plate.

To overcome these difficulties, Wagman and Bailey introduced Chrom-AR and silicic acid/glass fiber sheets for bioautography of antimicrobial compounds. The principle of the method was the same and antimicrobials had to be transferred from the chromatographic plates to agar causing their loss and dilution. Another special case is bioautographic detection of 6-aminopenicilanic acid which is a very weak antibiotic and must be converted through phenyl acetylation to benzyl penicillin by spraying chromatographic plates or paper with acetyl chloride in mild alkaline condition before bioautography (Betina and Pilatora 2000). This is a technique familiar to the microbiologists in search for antibiotics from microorganisms, and different procedures have been used to improve its performance. (Saikat *et al.,* 2015).

### 2.6.2 Direct TLC Bioautographic Detection

In direct TLC bioautography, the developed TLC plate is sprayed with or dipped into a fungal or bacterial suspension. A suspension of test bacteria or fungi is used for the spraying or dipping purpose. An inoculum of absorbance of 0.84 at 560 nm was suggested for bacteria like *Staphylococcus aureus* (Meyer and Dilika 2005), while using a suspension of 106 CFU/mL could be employed for both bacteria and fungi. The bioautogram is then incubated at 25 °C for 48 h under humid condition. For visualization of microbial growth, tetrazolium

salts are used. These salts are converted by the dehydrogenases of living microorganisms to intensely colored formazen (Silva *et al.*, 2005). These salts are sprayed onto the bioautogram and are reincubated at 25 °C for 24 h [28] or at 37 °C for 3–4 h (Dilika *et al.*, 2000). Clear white zones against a purple background on the TLC plate indicate antimicrobial activity of the sample (Tawari *et al.*, 2010). According to Saikat (2015), Oksua in 2010 incorporated a new medium for direct TLC bioautography which is fluid enough to disperse microorganisms and viscous enough to adhere to the TLC plates; according to them a mixture of Muller–Hinton (MH) broth and MH agar in the ratio of 90:10 fulfils this requirement. Homans and Fuch in 2000 found that direct spraying of the thin-layer chromatograms with a spore suspension of the test fungus in glucose–mineral salts medium was the easiest technique for the detection of fungitoxic substance, and it also gave the most reliable results . Direct bioautographic methods have been described for spore-producing fungi such as Aspergillus , Penicillium and Cladosporium (Homans and Fuch, 2000) and also for bacteria (Hamburger *et al.*, 2000) Bacillus subtilis , S. aureus and Escherichia coli are used to identify the active compounds. p- Iodonitrotetrazolium violet is the most suitable detection reagent (Burkhead *et al.*, 2005). TLC-direct bioautography is also useful for the rapid chemical and biological screening of plant extracts (Meyer *et al.*, 2015). Once an activity has been located at the TLC plate, the sample can be analyzed by liquid chromatography-mass spectrometry to establish whether known or new compounds and/or substance classes are involved. This screening strategy concerns rapid detection of antibacterial and antifungal compounds. Choma and Grzelak (2011) in their review on TLC- bioautography indicated plenty of examples of analysis on bacteria and fungi for the detection of antimicrobial activity of antimicrobial agent through bioautography and bioluminicence.

### 2.6.3 Immersion or agar overlay bioautography

Agar overlay is a combination of contact and direct bioautography. In this method, the chromatogram is covered with a molten, seeded agar medium. After solidification, incubation and staining (usually with tetrazolium dye) are performed. The inhibition or growth bands are visualized. (Marston, 2011). For Gram- negative bacteria, an agar solution containing the red-colored bacterium *Serracia marcescens* can be employed. The red-colored gel is incubated overnight at room temperature and inhibition zones appear as white or pale yellow areas on a red background (Willians *et al.*, 2001). With other, colorless, microorganisms, zones of microbial growth inhibition are visualized with the aid of a dehydrogenase activity-detecting reagent (tetrazolium salt). Metabolically active microorganisms convert the tetrazolium salt into the corresponding intensely colored formazan. The agar overlay assay has been used for yeasts such as *Candida albicans* and can also be applied to bacteria such as *B. subtilis* , *E. coli* , *Pseudomonas aeruginosa and S. aureus* . The antibacterial activity of isoflavonoid and sesquiterpenoid phytoalexins has been evaluated by an agar overlay method using *Pseudomonas syringae* pv. phaseolicola, with 2,3,5-triphenyl-tetrazolium chloride (TZC) as visualization reagent. Addition of glycerol to the overlay nutrient medium, as a carbon source, facilitated the reduction of TZC to pink colored formazans by bacterial dehydrogenases .

Antimicrobial carotenoids have been characterized in *Bixa orellana* seed extracts. The antibacterial activity was detected by overlaying TLC plates with agar containing *S. aureus* and then spraying with tetrazolium salt (INT) (). characterized eight antimicrobial peptides from Galleria mellonella larvae immune hemolymph using tricine SDS-PAGE bioautography. Several antimicrobial compounds were isolated from Tylosema esculentum husks, cotyledons, and tubers by employing agar overlay bioautography using *S. aureus, E.*

*coli, B. subtilis, P. aeruginosa and C. albicans* as test microorganisms. (Mazimba *et al.*, 2011)

# CHAPTER THREE

## 3.0 MATERIALS AND METHODS

### 3.1 Collection of Test Organisms

Referenced pure culture antibiotics resistant Coagulase Negative *Staphylococcus* strain (CoNs 10b: Ojo) and *Staphylococcus aureus (*W241: Ojo) were obtained from the Drug Discovery and Development Research (DDDR) unit, Department of Microbiology, Federal University Oye Ekiti.

### 3.2 Confirmation of Test Organisms

Colonies growing on slants were streaked on freshly prepared plates of Mannitol salt agar and incubated at $35^0$C for 24hours. Primary characterization of the isolates were based on Gram-staining and morphological characteristics, fermentation of Mannitol salt agar and other sugar fermentation (Lactose, Sucrose, Maltose, Fructose) Coagulase test, Catalase test and Citrate utilization test were performed as described by Ojo *et al* (2013).

### 3.3 Collection of Plant Sample- *Phyllanthus amarus*

The whole plant of *Phyllanthus amarus* was obtained from the environment of Federal University Oye Ekiti site and its environs with latitude of 7°8N and longitude of 5°33N. The harvested plant was washed thoroughly with distilled water and air dried.

### 3.4 Preparation of plant extract

The plant material obtained was washed with sterile distilled water and air dried at room temperature for about 2 weeks to ensure that the sample loose most of it moisture content. The dried plant material was then pulverized into powdered form using a household electric blender (Nakia magic, model number: S1-889BD). The powdered plant material was then

subjected to successive cold and hot extraction using n-Hexane, ethyl acetate and methanol to obtain the respective extracts. The hot extraction was done with a soxhlet apparatus (placed inside a water bath, this was to ensure extraction by steam in order to avoid destruction of the plant bioactive constituent that might be prone to heat) using 75g of the plant material in 500ml of n-Hexane, ethyl acetate and methanol respectively for a duration of 6 hours for extraction of each solvent. The cold extraction was carried out using 75g of the plant material in 500ml of n-Hexane, ethyl acetate and methanol respectively, which was covered for duration of 48hours for each solvents used. The extracts were filtered out using Whatman No. 1 filter paper and the obtained extracts were then concentrated using a rotary evaporator (Senco, model no: R205) at a speed of 39-40 rpm.

## 3.5 DETERMINATION OF BIOACTIVE BANDS USING THIN LAYER CHROMATOGRAPHY (TLC)

TLC was performed on a silica gel glass plates (200x200, 60 F254, Merck) to fractionate active components of the extract. The TLC plates were cut into 9.7x6.6cm sizes and dried in an oven at 90°C for 10 minutes, this is to activate the TLC plates by absorbing the moisture content from the plate.

### 3.5.1 Preparation of Mobile phase

Table 1. Mobile phase with different solvents ratio

| Solvent system | Composition | Ratio | Type of extract |
|---|---|---|---|
| i | Ethyl acetate: n-Hexane | 8: 2 | Ethyl acetate |
| ii | Methanol: ethyl acetate: n-Hexane | 4: 3: 3 | Methanol |
| iii | Methanol: ethyl acetate: n-Hexane | 4: 3: 3 | n-Hexane |
| iv | Methanol: ethyl acetate: n-Hexane | 3: 3: 4 | n-Hexane |

The plant extract were reconstituted in chloroform and a 5µl of extract was spotted at about 1cm apart and away from the bottom of the TLC plates, The plates were placed in ascending direction in a tightly enclosed jar with the different mobile system has developed in Table 1. The plates were visualized under the iodine fume in an enclosed chamber, the separated spots were marked and the retention factors ($R_f$) values were calculated.

### 3.6 Antibacterial Activity of Bioactive Compounds using Bioautography

Bioautography was done using agar overlay method; inoculums were prepared by suspending the microorganism in Mueller Hinton broth media with an approximate concentration of $10^6$cell/ml just before applying the overlay. The TLC plates were placed in sterile Petri dishes and covered with 4.5ml of seeded molten agar. The agar overlay plates were pre-incubated at room temperature and further incubation for 24 hours at 35°C. After incubation, the plates were sprayed with 1.0g/ml of aqueous solution of tetrazolium salt. The sprayed plates were re-incubated for 1h at 35°C, clear zones on the chromatograms indicating inhibition of growth were observed.

# CHAPTER THREE

## 4.0 RESULTS

### 4.1 Morphological and Biochemical Characterization of Referenced antibiotic resistant *Staphylococcus aureus* and *Coagulase negative staphylococcus aureus.*

The morphological and biochemical characterization of the referenced antibiotic resistant *Staphylococcus aureus* and *Coagulase negative staphylococcus aureus* revealed that the two strains are Gram positive, cocci and ferments various sugars with production of acid. (Table 2)

### 4.2 Determination of Bioactive bands using Thin Layer Chromatography (TLC)

The TLC analysis revealed the presence of some bioactive constituents in the plant *Phyllanthus amarus*. The results obtained indicate that maximum number of spots (8) for ethyl acetate extract was observed in the ethyl acetate: n-Hexane (8: 2) system with $R_f$ values (0.096, 0.137, 0.178, 0.260, 0.603, 0.644, 0.767, 0.838). In case of methanol extract maximum number of 5 spots were observed in Methanol: ethyl acetate: n-Hexane (4: 3: 3) with $R_f$ values (0.291, 0.473, 0.546, 0.655, 0.727), while in n-Hexane extract maximum numbers of spots were seen in Methanol: ethyl acetate: n-Hexane (4: 3: 3) with $R_f$ values of 0.741, 0.724 (Table 3).

### 4.3 Antibacterial Activity of Bioactive Compounds using Bioautography

The appearance of white area against a purple-red background on the chromatograms denotes inhibitions of growth of the microorganism due to the presence of compounds that inhibits their growth. Actively growing microorganism has the ability to reduce INT (2-(4-iodophenyl)-3-(4-nitropheynl)-5-phenyl-2H-tetrazolium chloride) in the tetrazolium salt to a purple-red color. In the presence of bioactive plant constituents on the chromatograms, the

growth of the organism is inhibited. The results obtained in Table 4 shows that the ethyl acetate extract which retained at $R_f$ value 0.835 shows 21mm zone of inhibition which is the maximum zone of inhibition against coagulase negative *Staphylococcus*, while the compound which retained at $R_f$ value of 0.835 shows no zone of inhibition against *S. aureus*. For methanol extract the compound which retained at $R_f$ value of 0.727 was observed to show 23mm zone of inhibition which is the maximum zone of inhibition against coagulase negative *Staphylococcus*, while the compound which retained at $R_f$ value of 0.727 for methanol extract against S.*aureus* shows no zone of inhibition. For n-Hexane extract the compound which retained at $R_f$ value of 0.741 shows 21mm zone of inhibition which is the maximum zone of inhibition to *Staphylococcus aureus*, while the compound which retained at $R_f$ value of 0.741 shows no zone of inhibition against Coagulase negative *Staphylococcus* (Table 4).

**Table 2. Morphological and Biochemical Characterization of Referenced isolates**

| Test organism | Shape | Gram | Coagulase test | Catalase test | sugar fermentation test | | | | Citrate utilization test |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Lactose | sucrose | Maltose | Fructose | |
| **CoNS 10b: Ojo** | **Cocci** | **+ve** | **+ve** | **+ve** | **A** | **AG** | **A** | **AG** | **+ve** |
| ***S. aureus* W241: Ojo** | **Cocci** | **+ve** | **-ve** | **+ve** | **A** | **A** | **AG** | **AG** | **+ve** |

**KEYS:** +ve: gram positive, -ve: negative, A: acid, AG: Acid & gas production

**Table 3. Retention factor ($R_f$) of *Phyllanthus amarus* in different solvents compositions**

| Extract | Solvent system | Numbers of bands | Active bands | $R_f$ of each spots |
|---|---|---|---|---|
| Ethyl acetate (Hot) | E: n-H (8:2) | 6 | 5 | 0.189, 0.649, 0.676, 0.716, 0.757, 0.835 |
| Ethyl acetate (Cold) | E: n-H (8:2) | 8 | 7 | 0.096, 0.137, 0.178, 0.260, 0.603, 0.644, 0.767, 0.838 |
| Methanol (Hot) | M:E: n-H (4:3:3) | 5 | 4 | 0.291, 0.473, 0.546, 0.655, 0.727 |
| Methanol (Cold) | M:E: n-H (4:3:3) | 3 | 2 | 0.40, 0.60, 0.727 |
| n-Hexane (Hot) | M:E: n-H (4:3:3) | 4 | 3 | 0.483, 0.569, 0.655, 0.724 |
| n-Hexane (Cold) | M:E: n-H (4:3:3) | 4 | 3 | 0.517, 0.604, 0.690, 0.741 |

**KEYS:** E-n-H= Ethyl acetate: n-Hexane, M: E: n-H= Methanol: Ethyl acetate: n-Hexane

**Table 4. Zones of inhibitions on Direct Bioautographic Plates of *Phyllanthus amarus* Extract.**

| Extracts | Diameter of zone of inhibition on CoNS (mm) | Diameter of zone of inhibition on *S.aureus* |
|---|---|---|
| Ethyl acetate (Hot) | 21mm | Nill |
| Ethyl acetate (Cold) | 18mm | Nill |
| Methanol (Hot) | 23mm | Nill |
| Methanol (Cold) | 21mm | Nill |
| n-Hexane (Hot) | Nill | 19mm |
| n-Hexane (Cold) | Nill | 27mm |

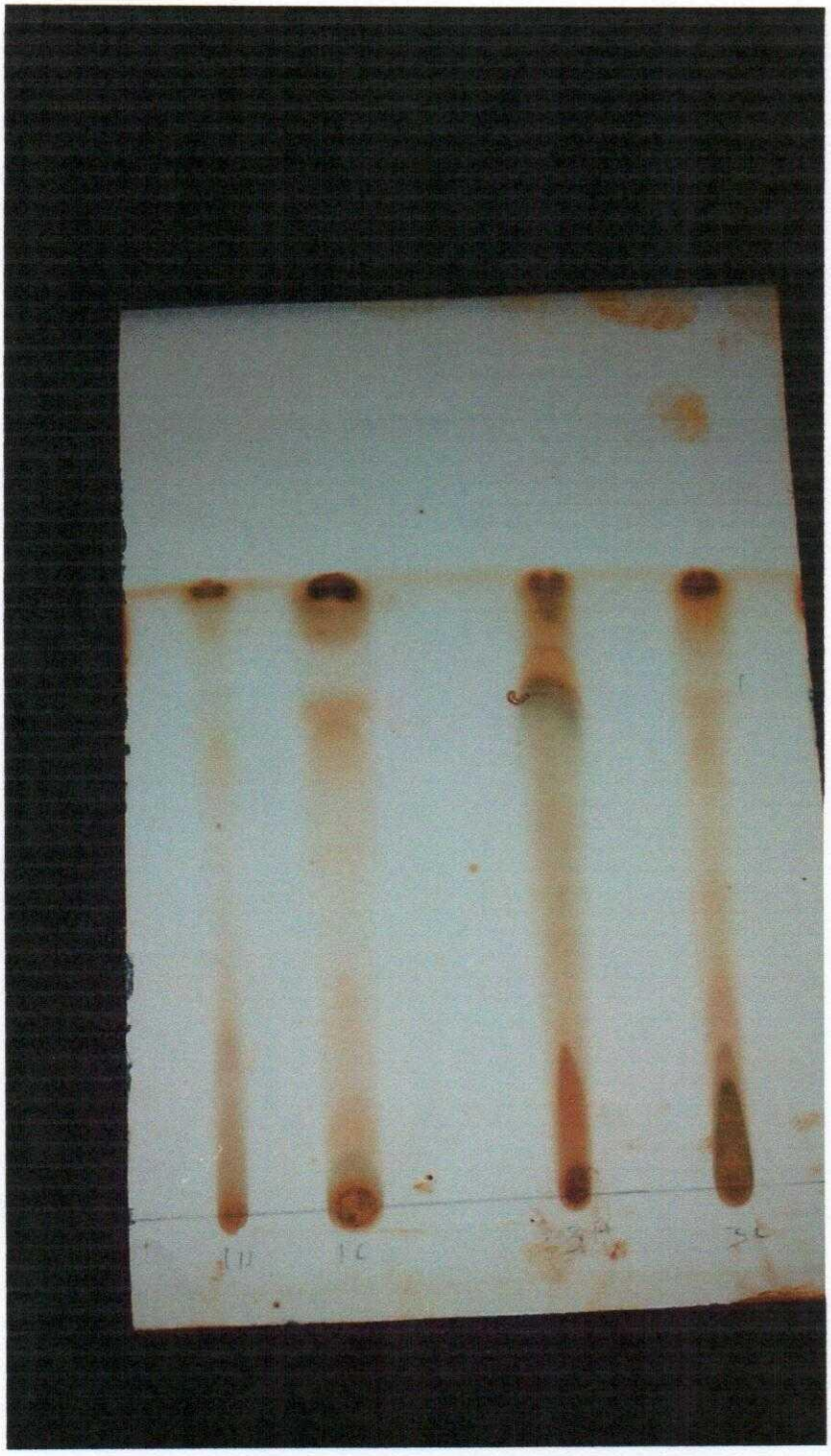

**Plate 2. Active bands of ethyl acetate extract on TLC plate in Ethyl acetate: n-Hexane (8: 2) solvent system**

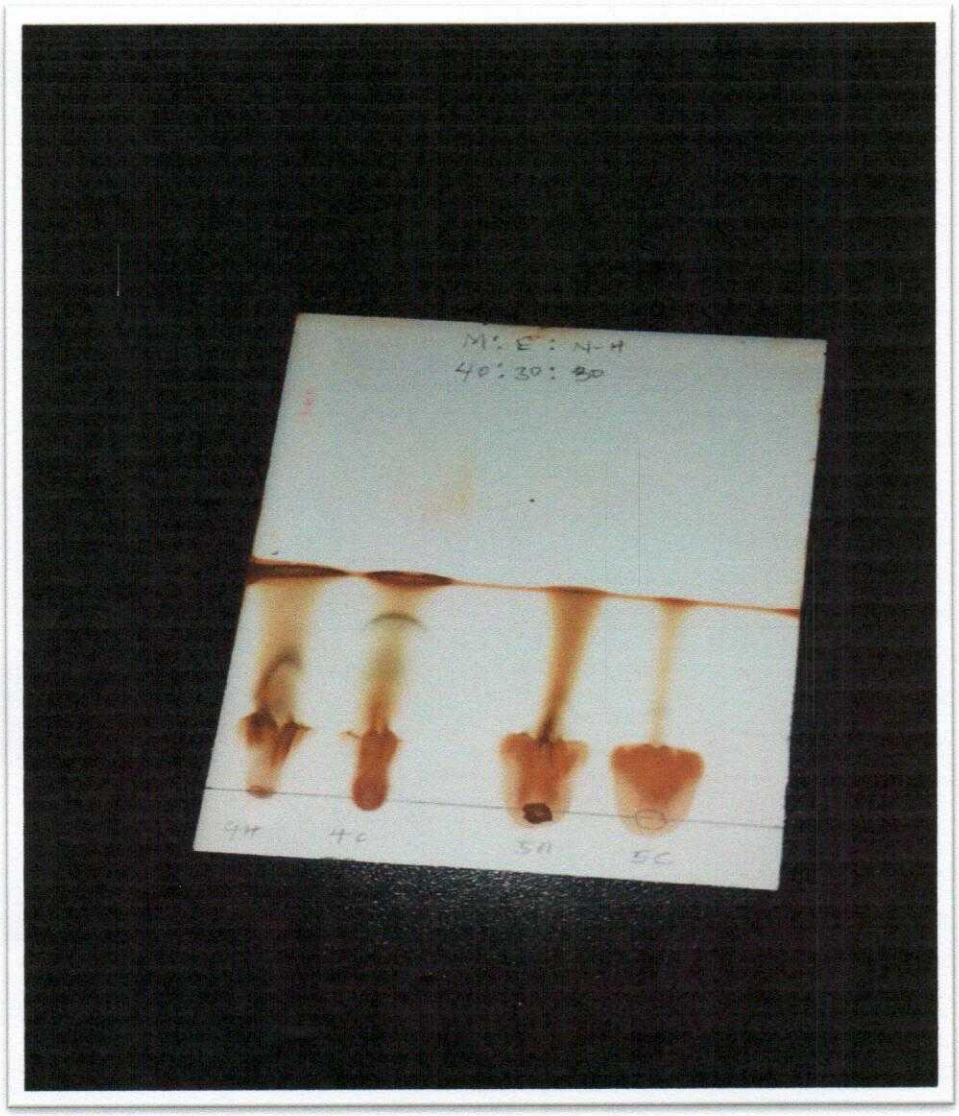


**Plate 3. Active bands of methanol extract on TLC plates in Methanol: Ethyl acetate: n-Hexane solvent system**

**Plate 4. Photograph showing zones of inhibition on ethyl acetate of *Phyllanthus amarus* chromatogram**

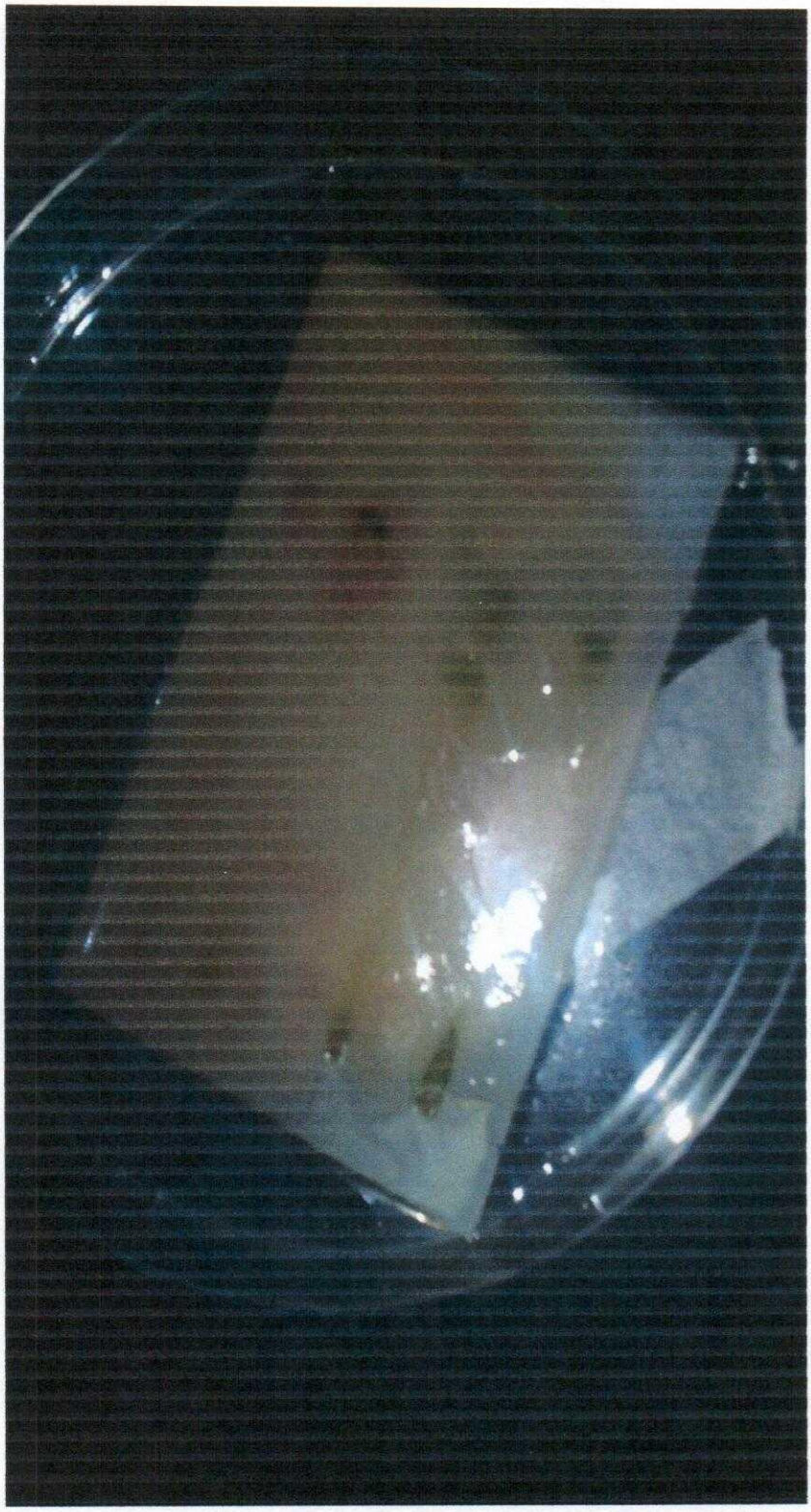

**Plate 5. Photograph showing zones of inhibition on n-Hexane of *Phyllanthus amarus* chromatogram**

# CHAPTER FIVE

## 5.0 DISCUSSION

### 5.1 Morphological and Biochemical Characterization of Referenced antibiotic resistant *Staphylococcus aureus* and *Coagulase negative staphylococcus aureus.*

The activeness of the referenced strains were confirmed from the result in Table 2 showing Gram positive cocci, Coagulase positive and fermentation on mannitol which indicates *S. aureus,* while strains showing Gram positive cocci, Coagulase negative and non-fermentation on mannitol indicates Coagulase negative *S. aureus* which was similar to a report by Ojo *et al* (2013).

### 5.2 Determination of Bioactive bands using Thin Layer Chromatography (TLC)

In similarity with the report by Abah and Egwari (2011) cold extracts of Ethyl acetate and n-Hexane in this study shows to have more active bands of phytochemical constituents than hot ( soxhlet) crude extract, but in contract cold extracts of methanol from this study shows to have more active bands of phytochemical constituents than hot (soxhlet) crude extract, thus indicating that for ethyl acetate and n-Hexane extracts more active phytochemicals are better extracted from cold percolation method than soxhlet extraction method which is not the case for methanol.

### 5.3 Antibacterial Activity of Bioactive Compounds using Bioautography

Table 4 shows zones of inhibition of plant extracts on *S. aureus* and Coagulase negative *Staphylococcus aureus.* N-Hexane cold extraction showed highest zone of inhibition (27mm and 19mm for cold and hot extracts respectively) while methanol extraction has no zone of inhibition on *Staphylococcus aureus*, which was similar to the report by Mushore and

Matuvhunye (2013) and contradict the report of Middha and Parihar (2012). This shows maximum zone of inhibition of 37mm against Methicillin Sensitive S.aureus with methanol extract.

# 6.0 CONCLUSION AND RECOMMENDATION

## 6.1 CONCLUSION

Data from literatures as well as the present study results revealed the great potential of *Phyllanthus amarus* extracts for therapeutic treatment. Although some experience is required to obtain a good bioautograms, the method is very useful in isolating compounds with antimicrobial activity. In this study, *Phyllanthus amarus* extract has shown its efficacy against *Staphylococcus aureus* and Coagulase negative *Staphylococcus*.

## 6.2 RECOMMENDATION

This study shows that the bioactive constituents present in *Phyllanthus amarus* extracts exhibits antibacterial activity. Therefore it is recommended that further studies should be carried out to identify the specific constituents. Other bioautographic methods should also be carried out and tested for comparison with more solvent system variations. Toxicity test can also be carried out to reveal side effect of the extract.

## 6.3 CONTRIBUTION TO KNOWLEDGE

1. Active band sites on the TLC plates of *Phyllanthus amarus* extracts was high in ethyl acetate and methanol extracts (7-4), than n-Hexane extract with (3-1).

2. Zone sizes of inhibition ranged between 18mm -27mm.

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## APPENDIX

## MORPHOLOGICAL TEST

### a) Gram staining

**Materials**

Crystal violet, iodine, ethanol, saffranin, running water, inoculating loop, clean and sterile glass slide, Bunsen burner, microscope.

**Procedure**

A thin smear of cell suspension was made o a clean glass slide and then heat fixed. The smear was then stained with a drop of crystal violet for about one minute after which it was rinsed mildly with running water. A drop of iodine was then added for one minute ( the iodine decreases the solubility if the purple dye forming the dye-iodine complexes) which was also rinsed mildly with running water. The purple dye-iodine complex were then decolorized with 95% ethanol for 15seconds and rinsed. The smear counter stained with saffranin for 30 seconds. The excess stain was rinsed off with water. Smeared slide was then air dried. A drop of oil immersion was added to the slide for clearer viewing under the microscope. The slide was then viewed under the microscope.

**Result**

Purple colour observed indicates a Gram positive result while pink colour indicates a Gram negative result.

## BIOCHEMICAL TEST

### a) Catalase test

**Materials**

1% hydrogen peroxide, inoculating loop, clean glass slide, Bunsen burner

**Procedure**

A colony of organism was picked and placed in 1% hydrogen peroxide on a clean sterile glass slide

**Result**

Effervescence caused by production of oxygen as gas bubble indicates a catalase positive result while absence of bubble indicates a negative result.

### b) Coagulase

**Materials**

Blood plasma, normal saline, inoculating loop, clean glass slide, Bunsen burner

**Procedure**

A colony of cell suspension was emulsified with normal saline on a sterile glass slide, an equal amount of blood plasma was added to the mixture and mixed together.

Result

presence of clumps in the mixture indicates a coagulase positive result while absence indicates a negative result.

**c) Citrate test**

A colony of the sample organism was picked and inoculated into Simmons citrate agar and incubated at 35°C. growth in the agar causing color change from green to blue indicates a positive result.