INHIBITORY POTENTIAL OF LEAF AND ROOT BARK EXTRACTS OF *TERMINALIA AVICENNIOIDES* ON METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* AND CHARACTERIZATION OF THE DISTINCT BIOACTIVE EXTRACT

BY

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DECLARATION

I declare that the work in this Dissertation entitled *"*Inhibitory Potential of Leave and Root Bark Extracts of *Terminaliaavicennioides*on Methicillin Resistant *Staphylococcus aureus* and Characterization of the Distinct Bioactive Extract*"* has been carried out by me in the Department of Biochemistry. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other Institution.

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CERTIFICATION

This Dissertation entitled *"*Inhibitory Potential of Leave and Root Bark Extracts of *Terminaliaavicennioides*on Methicillin Resistant *Staphylococcus aureus* and Characterization of the Distinct Bioactive Extract*"* by EkeneUgochukwu ADIM, meets the regulations governing the award of the degree of Master of Science of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This dissertation is dedicated to my parents, Mr. and Mrs.UcheAdim, and my Siblings. They not only shared every step of my study and researchprogress, but also are always the source of strength, support and encouragement.

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ABSTRACT.

The increasing concern of antibiotic resistance has lead to search for new antibiotic chemotheraphy. The aim of this study was to investigate the Inhibitory potential of the leaf and root bark extracts of *Terminaliaavicennioides* against Methicillin resistant *Staphylococcus aeurus* (MRSA) and characterization of the most distinct bioactive extract.Extraction was done using n-hexane, Chloroform, Ethyl acetate and Methanol. The extracts were graded at concentrations of 60µg/ml, 120µg/ml, 180µg/ml and 240µg/ml. With exception of chloroform, ethyl acetate and n-hexane extract of the leaf at 60µg/ml, every other extracts of the leaf and root bark at all concentrations are inhibitory to the growth of MRSA within the susceptible range ≥ 14mm as defined by Kirby-Bauer standard chart for (Methicillin) antibiotic susceptibility. Chloroform crude extract of the leaf and Ethyl acetate crude extract of the root bark were observed to be the best system for isolation of bioactive principles. A comparison of activity of these two lead to further investigation of Ethyl acetate extract of the root bark. TLC fractionation was done using a mixture of Chloroform: Ethyl acetate: Methanol: Water (15:8:4:1) with a resultant 18 fractions (bands). The organism showed a mean zone inhibitory sensitivity of 14mm at 60µg/ml, 15.76mm at 120µg/ml and 15.33mm at 180µg/ml for fraction TLb4 and 15.33mm at 60µg/ml, 17.33mm at 120µg/ml and 20mm at 180µg/ml for TLb17 (≥ 14mm). The two Fractionswhere subjected to GC-MS and FTIR analysis. Minimum Inhibitory Concentration was 0.6µg/ml for both TLb4 and TLb17 and as well bactericidal at this same concentration. Phytochemical screening of TLb1 to TLb18 fractions from ethyl acetate extract shows that terpenoidswhere present in all fractions. Quantitatively, 42µg of alkaloids, 19.80µg of anthraquinone and 184.25µg of tannins were estimated in 1mg of ethyl acetate root bark extract.Analysis of GC-MS revealed that the most likely

pharmacological compounds present in both fractions were oleic acid, analogs of palmitic acid (n-Hexadecanoic), methyl ester (11-Octadecenoic acid), stearic acid (Octadecenoic acid) in association with a possible synergistic effect of 9-Octadecenal, 3,11-Tetradecadien-1-ol, and (9Z)-9-Teradecenal present only in fraction TLb17. FTIR shows the presence of Alkyl Halides: R-Br, R-F, Methyne: (CH−), Nitro Compounds:R-NO_2, Amides: R-C(O)-NH2, R-C(O)-NH-R., Isothiocyanate: (-NCS), Nitril: C≡N, Carboxylic Acids, Saturated Aliphatic (Alkyl):METHYLENE C-H, Alkanes, in both TLb4 and TLb17, and Alkyl Halide: R-I, Allenes, Amines: R-NH2 in TLb17 only as functional groups. These bioactive compounds and functional groups could be effective therapeutic agents for the treatment and management of infections caused by MRSA.

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ABBREVITION, DEFINITION OF TERMS, GLOSSARRIES AND SYMBOLS

ATCC American Type Culture Collection

ANOVA Analysis of Variance

CA-MRSA Community-Associated Strains of Methicillin-Resistant *Staphylococcus aureus*

ccr Cassette Chromosome Recombinase.

CDC Centers for Disease Control

CDC&P Centers for Disease Control and Prevention

CLLSICinical and Labouratory Standard Institute

DMSO Dimethyl sulfoxide

DNA Deoxyribonucleic acid

FTIR Fourier Transform Infrared Spectroscopy

GC-MS Gas Chromatography Mass Spectroscopy

HAMRSAHospital-Associated Strains of Methicillin-Resistant *Staphylococcus Aureus*

ICUs Intensive Care Units

ILSI International Life Science Institute

MBC Minimum Bactericidal Concentration

MDR Multidrug Resistant

MIC Minimum Inhibitory Concentration

MRSA Methicillin Resistant Staphylococcus Aureus

MSSA Methicillin Susceptible Staphylococcus Aureus

NHIS National Healthcare-Associated Infections Surveillance

NNIS National Nosocomial Infection Surveillance

orfX Open reading frame X

PBP		Penicillin Binding Protein
PBP-2a		Penicillin Binding Protein 2a
PCR		Polymerase Chain Reaction
SCCmec		Staphylococcal Chromosome Cassette
SSTIs		Skin and Soft Tissue Infections
ST		Sequence type
TLC		Thin Layer Chromatography
%	=	Percentage
µg	=	Microgram
mg	=	Milligram
g	=	gram
ml	=	Mililiter
mm	=	Milimeter

GLOSSARRIES

Cassette Chromosome Recombinase***(ccr)*:**A gene necessary for the mobility of SCC that enables its site-specific integration into and precise excision from the *S. aureus* chromosome.

Genomic Island**:**Genomic islands (often abbreviated as GIS or GEIs) are horizontally acquired chromosomal regionsof DNA carrying several genes encoding traits associated with increased adaptability or fitness under specific conditions. They are termed pathogenicity, fitness, symbiosis, metabolic, or resistance islands, depending on the functions encoded.

Housekeeping Gene**:**A gene involved in basic functions required for cell viability and constitutively expressed in mostcells. Housekeeping genes evolve much more slowly than do tissue specific genes that encode proteins necessary only inselected types of cells.

Insertion Sequence**:**A DNA sequence involved in the mobilization of genetic information to and from vectors such asplasmids.

*mec*gene Complex**:**Gene complex composed of *mecA*and its regulator genes, *mecI*and *mecR.*

*mecA***:**The gene encoding PBP2a, responsible for resistance to methicillin and other β-lactam antibiotics.

*mecC*gene**:**A novel resistance gene in methicillin-resistant Staphylococcus aureus (MRSA). It is a homologue to*mecA.* It poses diagnostic problems with the potential to be misdiagnosed as methicillin-sensitive S. aureus, with important potential consequences for individual patients and for the surveillance of MRSA. It was formally called mecALGA251 because it is of a *S. aureus* isolate, it also confers resistance to oxacillin and cefoxitin).
*mecI*_GENE: The *mecA*repressor gene. It is a gene that encodes a transcriptional regulator (mecA_GENE_repressor) which is a strong repressor of the expression of the *mecA* gene.

*mecR1*_GENE: A signal transducer gene that encodes a cytoplasmic transmembrane-bound signal transduction receptor protein (mecR1_PROTEIN) that responds to covalent binding of a blactamantibiotic and its extracellular sensor domain. Binding initiates events that lead to inactivation of the *mecI*gene repressorproduct by a protease, allowing expression of *mecA.*

Staphylococcal Chromosome cassette (SCC) or (SCC*mec*):Is a mobile, 52-kb DNA cassette containing the genethat encodes resistance to methicillin *(mecA)*, as well as those genes (*ccrA*and *ccrB*in most cases) that encode the integration and excision necessary for its recombination in the staphylococcal chromosome, in addition to insertion sequences.

CHAPTER ONE

1.0 INTRODUCTION

Methicilin resistant *Staphylococcus aureus* strains are prevalent bacterial pathogens that cause both health care and community-associated infections and over the years community-associated strains of methicillin-resistant *Staphylococcus aureus* (CA-MRSA) have emerged and spread rapidly, accounting for large increases in disease both in the community and in the hospital (Klein *et al.*, 2007; Chambers and DeLeo, 2009). Increasing resistance to commonly prescribed antibiotics like methicillin has made these antibiotic resistant strains of *Staphylococcus aureus,* a serious threat to public health throughout the world.Ever since the gram-positive bacterium *Staphylococcus aureus* was first discovered by Sir Alexander Ogston in 1880, it has been regarded as a serious threat to human health, capable of causing a multitude of infections. Initially, MRSA strains afflicted hospitalized patients and those with chronic illnesses. But the 1990s saw the emergence of community associated MRSA (CA-MRSA) strains that primarily caused skin and soft tissue infections (SSTIs) in otherwise apparently healthy individuals. These strains quickly led to an epidemic of CA-MRSA infections including some with severe consequences, for example, community-acquired pneumonia with high mortality rates (Francis *et al.,* 2005), pyomyositis (Burdette *et al.,* 2012), sepsis, osteomyelitis (Kechrid *et al.,* 2011) and necrotizing fasciitis (Changchien *et al*., 2011).

Methicillin resistance is mediated by PBP-2a, a penicillin binding protein encoded by the mecA gene that permits the organism to grow and divide in the presence of methicillin and other beta-lactam antibiotics. The mecA gene is located on a mobile genetic element called staphylococcal chromosome cassette (SCCmec) (Inglis *et al.,* 1988).

Prevalence in some Nigerian cities shows that out of 98 (49 %) *S. aureus isolated* from 200 nasal swab samples in Jos Nigeria, Sixty two isolates (63.3 %) were found to be methicillin resistant while 55 (88.7 %) of the methiciln resistant*Staphylococcusaureus* (MRSA) isolated were multidrug resistance and only 3 (4.8 %) were susceptible to all other tested antibiotics(Ajoke *et al.*, 2012).In a total of 54 (36%) *S. aureus* isolated from 150 urine samples of women in Zaria Nigeria, 37 (69%) were methicillin-resistant. All the 37 (100%) MRSA isolates showed resistance to at least two antibiotics tested while 33 (89.2%) were multi-drug resistant (Onanuga*et al*., 2005).

Antibiotic failure is due to the appearance of multidrug resistant pathogens and the spread of the new infections (Abdalla, 2011). Example is methicillin resistant *S. aureus* (MRSA); a bacterium that has developed resistance to most antibiotics such as methicillin, gentamycin, fucidic acid and clindamycin that are commonly used for *Staphylococcus* infections, unfortunately, leading to failure of treatment (Shai *et al.,* 2004). The effectual lifespan of an antibiotic is limited and over-recommendation and mistreatment with traditional antibiotics have caused microbial resistance to develop (Alam *et al.,* 2009). This increasing frequency of resistance to most antimicrobial agents complicates their use and the control of infectious diseases. This situation seems to pose the search for the use of antimicrobial drugs in future to be uncertain (Nascimento*et al.,* 2000); as a result, the search for new ways to treat MRSA infections stimulates research into natural products from medicinal plants (Aliyu *et al.,* 2008).

A plant-based system continues to play an essential role in healthcare. Herbs have been utilized by people throughout history (Tagboto and Townson, 2001). A large variety of

secondary metabolites are produced by medicinal plants; these are either used as precursors or lead compounds in the pharmaceutical industry and it is expected that medicinal plant extracts showing target sites other than those used by antibiotics will be active against drug-resistant microbial pathogens. (Shokeen *et al.,* 2009).

The use of medicinal plants has a long history, especially, in Africa. It is estimated that two-third of the world population rely on traditional medical remedies due to the limited availability and affordability of pharmaceutical products (Tagboto and Townson, 2001).

Terminalia avicennioides is a yellowish brown, hard and durable wood. It belongs to the family Combrataceae, genus *Terminalia,* and species *avicennioides*. In Nigeria, it is called baúsheè in HausaBárbár, in Kanuri,Kpace in Nupe, Kuegh inTiv, Idi inYorubaand Edo in Igbo. The root, which is used as chewing sticks have been claimed to cure dental caries and skin infections (Gill and Akinwunmi, 1986). Previous studies showed that the bark extract of *T. avicennioides* exhibited both vibrocidal and typhoidal activities (Akinside and Olukoya, 1995; Akinyemi *et al.,* 2000).

Traditionally,in different localities in the Northern part of Nigeria, the root bark extract of *T. avicennioides* are drank and bathed with and in some cases are mixed with local creams in the treatment of skin infections . Traditionally alsoextracts of the stem bark and roots of *T. avicennioides* are used for the treatment of bacterial infections such as gastrointestinal disorders (Abdullahi *et al*., 2001), syphilis (Irvine, 1961), dental caries and skin infections (Lewis and Elvin-Lewis, 1977); bloody sputum (tuberculosis) and cough in humans (Mann *et al*., 2007a). Pharmacologically, crude methanolic extract of the *T. avicennioides* root bark exhibited broad growth inhibition against microbes causing infectious diseases (Mann *etal*., 2007b).

1.1STATEMENT OF RESEARCH PROBLEM

Infectious diseases with increasing trends of drug resistant microorganisms have been common global problem posing enormous public health concerns (Iwu *et al*., 1999).

In the hospital, *S. aureus* is the most frequent cause of surgical; lower respiratory tract, and cardiovascular infections. Furthermore, it is the second most common cause of health care-associated pneumonia and bloodstream infections (Loffler and Macdougall, 2007; Richards *et al.,* 1999a; Richards*et al.,* 1999b).

Report by Center for Desease Control and Prevention indicates that in 1999, MRSA accounted for >50% of *S. aureus* isolates from patients in intensive care units (ICUs) in the National Nosocomial Infection Surveillance (NNIS) system (CDC&P, 2003); in 2004 the proportion of MRSA isolates was reported to have increased from 59.5% to 64.4% in ICUs in the United States (CDC&P, 2004; Klevens *et al*., 2006). Hospital surveillance data in 2001 also showed that MRSA was the biggest threat in China, where resistance among hospital-acquired infections reached almost 90% (Zhang *et al*., 2006).

The global emergence of antimicrobial resistant bacterial strains is increasingly limiting the effectiveness of current drugs and causing treatment failure of infections (Hancock, 2005).

In many poor countries, the available drugs are costly and beyond the reach of the common man (WHO, 2002); as a result, the search for new ways to treat MRSA infections stimulates research into natural products from medicinal plants.Hence to investigate the inhibitory potential of*Terminalia avicennioides* leave and root bark extracts on methicillin resistant *Staphylococcus aureus,* and to determine the active component that doesthe

inhibition in the distinct bioactive extract is a necessity towards solving the health problem associated with methicilline resistant*Staphylococcus aureus*.

1.2 JUSTIFICATION FOR THE STUDY

Indigenous plants are reservoirs of various metabolites and provide a limitless source of important chemicals that have diverse biological properties and many of modern day drugs have their origin in traditional plant medicine and the screening of plant extracts for antimicrobial activity has shown that plants represent a potential source of novel antibiotic chemotherapy.

Most therapeutic attributes of medicinal plants are traced to the plant constituents and the medicinal actions of these constituents are unique to particular plant part, species or family. Hence the use of the leave and roots bark of *Terminalia avicennioides*

Traditionally, the roots bark extract of *Terminalia avicennioides* are drank, bathed with and in some cases are mixed with local creams in the treatment of skin infectionsin different localities in the Northern part of Nigeria.

Theindigenous useof*Terminalia avicennioides* and in-vitro anti-methicilline resistant *Staphylococcus aureus* activity of *Terminalia avicennioides* has been reported by (Akinyemi *et al.*,2005), however fractionation and characterisation of the bioactive component of the extract was not investigated.

1.3 Aim and Objectives

1.3.1Aim

The aim of this study is to evaluate the inhibitory potential of the leaf and root bark extracts of *Terminalia avicennioides*against methicillin resistant *Staphylococcus aureus* and characterization of the distinct bioactive extract.

1.3.2Specific Objectives

This research is designed to achieve the following specific objectives:

i. Determine the most bioactive crude extract between the leaf and root bark extracts of *Terminalia avicennioides*against methicillin resistant *S. aureus*and evaluation of phytochemical content of the most active crude extract.

ii. Isolate the bioactive compound (s) of the most bioactive crude extract of *Terminalia avicennioides*, by preparative TLC, determine their inhibitory effect against MRSAand evaluate the phytochemical contents.

iii. Determine the Minimum Inhibitory ConcentrationandMinimum Bactericidal Concentration of the bioactive TLC band(s) of the most bioactive extract of *Terminalia avicennioides*against MRSA.

iv. Characterizethe compounds in the bioactiveTLC band(s) by GC-MSanddetermine the functional groups present in the compoundsby Infrared spectroscopy.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 History of *Staphylococcus aureus* and Methicillin-resistant *Staphylococcus aureus*(MRSA)

Staphylococcus aureus are gram positive, non-motile, and perfectly spherical, measuring approximately 1 micrometer. *S. aureus* grow in chains and resemble bunches of grapes. The unusual name is derived from the Greek staphyle, meaning "bunch of grapes". When observed microscopically in stained specimens taken from colonies grown on blood agar, *S. aureus* strains grow out a hemolytic pattern.*S. aureus* also display golden color of colonies when grown on agar aerobically. *S. aureus*was first described at the end of the 19th century in pus from human abscesses. Itis a major pathogen that is responsible for not only severe infections of the skin and skin structures but also life-threatening diseases because of its propensity to form biofilms on artificial materials, difficult-to-treat infections of catheters and other devices (Moreillon *et al.*, 2005) *S. aureus* was certainly a significant human pathogen prior to the development of antibiotics. Following the introduction of antibiotics, *S. aureus* developed resistance to penicillin in the 1947, just four years after the drug started being mass-produced and then emerged as an important cause of serious nosocomial infections in the 1950s. Methicillin was then the antibiotic of choice, but has since been replaced by oxacillin due to significant kidney toxicity. With the development and widespread use of chloramphenicol and tetracycline in the 1960s, superinfections due to *S. aureus* occurred including staphylococcal enterocolitis (Steves, 2007). The incidence of *S. aureus* related infections has increased dramatically since the emergence of methicillin resistant strains and high rates of mortality and morbidity are occurring worldwide (Oliveira *et al.*, 2002). Infections caused by *S. aureus* range from mild (pimples

or boils) to life-threatening ones such as infection of the bloodstream, heart valves, pneumonia, bones or joints. It is experimentally reported that benign appearing skin and soft tissue infections caused by MRSA canprogress rapidly to potentially fatal diseases (Olsen *et al*., 2008).

2.1.1 Methicillin and Other Drug Resistant *Staphylococcus aureus*

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of infections in healthcare institutions (Panlilio *et al*., 1992) and more recently in the community (Drews *et al.,* 2006). MRSA was first reported in 1961 in Britain, two years after the introduction of methicillin in 1959 as the first beta-lactamase-resistant penicillin for the treatment of penicillin-resistant *S. aureus* infections (Enright *et al.,* 2002). Despite extensive infection control efforts, methicillin resistance among isolates of *S. aureus* has steadily increased. What is really worrying are the information with report of patients that have been infected with strains of *S. aureus* resistant to methicillin and vancomycin having also acquired the ability to release cellular toxins (O'Hara *et al*., 2008). This is raising the spectre of the worst kind of antibiotic-resistant superbug (O'Hara *et al*., 2008).Rates of invasive infections with methicillin-resistant S. aureus (MRSA) have increased both in the hospital and in the community. The prevalence of methicillin-resistant *S. aureus* (MRSA) worldwide (Tiemersma, 2004) is continuously increasing. Data from the National Healthcare-associated Infections Surveillance (NHIS) system of the Centers for Disease Control and Prevention (CDC) show that 50% of healthcare-associated *S. aureus* isolates are now resistant to methicillin (Safdar *et al.,* 2007).

MRSA is common in long-term care facilities. Described in the popular media as "superbugs," MRSA organisms generally are resistant to multiple antibiotics, including

aminoglycosides, macrolides, fluoroquinolones, clindamycin, trimethoprim-sulfamethoxazzole, chloramphenicol, and beta-lactams.

Humans are a natural reservoir for *S. aureus*, (Chambers, 2001) and the anterior nares and skin are the usual sites of MRSA colonization, (Coello *et al*., 1994) which may persist for years. (Sanford *et al*., 1994 and Eveillard *et al*.,2004). In health care facilities, inanimate surfaces are occasional sources of nosocomial spread (Boyce *et al*., 1997), although patient-to-patient is the most common MRSA route of transmission, and the contaminated hands or gloves of health care workers usually serve as intermediate transmission vectors (Coello *et al*., 1994,Sanford *et al.,* 1994 and Eveillard *et al.,* 2004). Thus, infection control policies that include isolation and barrier precautions are critical components of prevention.

2.1.2 Antibiotic resistance and Mechanism of Antibiotic Resistance

Antibiotic resistance is a form of drug resistance whereby some (or, less commonly, all) sub-populations of a microorganism, usually a bacterial species, are able to survive after exposure to one or more antibiotics; pathogens resistant to multiple antibiotics are considered multidrug resistant (MDR) orcolloquially, superbugs. (Ijeh and Ukweni, 2007).

Several mechanisms have evolved in bacteria which confer them with antibiotic resistance. These mechanisms can chemically modify the antibiotic, render it inactive through physical removal from the cell, or modify target site so that it is not recognized by the antibiotic. The most common mode is enzymatic inactivation of the antibiotic. An existing cellular enzyme is modified to react with the antibiotic in such a way that it no longer affects the microorganism. An alternative strategy utilized by many bacteria is the alteration of

theantibiotic target site. These and other mechanisms are shown in the figure 2.1on the next page.

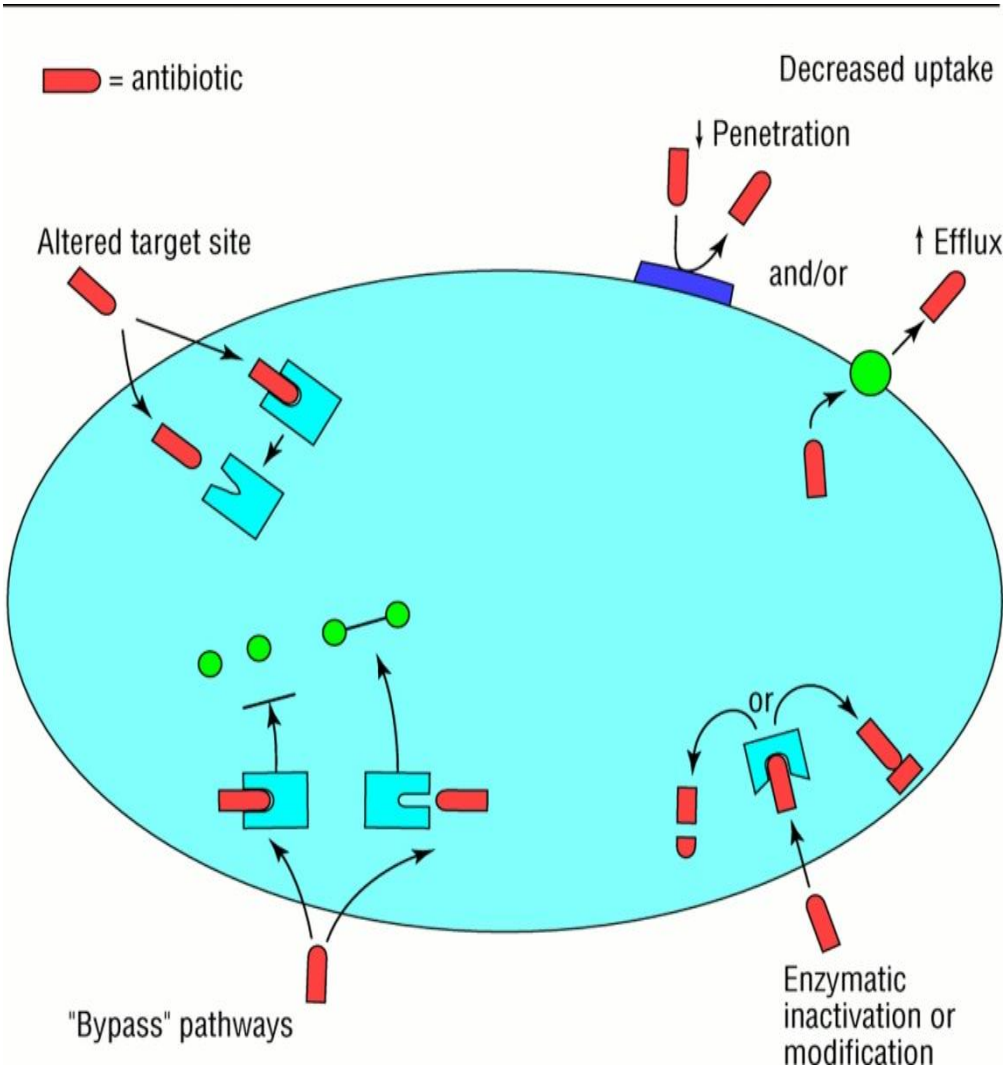


Figure 2.1: Bacterial Resistance Mechanisms on Antibiotics.Adapted from Hawkey, (1998).

Antibiotic resistance is a serious and growing phenomenon in contemporary medicine and has emerged as one of the pre-eminent public health concerns of the 21st century; particularly as it pertains to pathogenic organisms (the term is especially relevant to organisms which cause disease in humans). In the simplest cases, drug-resistant organisms may have acquired resistance to first-line antibiotics, thereby necessitating the use of second-line agents.

Typically, a first-line agent is selected on the basis of several factors including safety, availability and cost; a second-line agent is usually broader in spectrum, has a less favorable risk-benefit profile and is more expensive or, in dire circumstances, is locally unavailable.

In the case of some MDR pathogens, resistance to second and even third-line antibiotics is thus sequentially acquired; a case quintessentially illustrated by *Staphylococcus aureus* in some nosocomial settings. Some pathogens, such as *Pseudomonas aeruginosa*, also possess a high level of intrinsic resistance.

It may take the form of a spontaneous or induced genetic mutation or the acquisition of resistance genes from other bacterial species by horizontal gene transfer via conjugation, transduction, or transformation. Many antibiotic resistance genes reside on transmissible plasmids, facilitating their transfer. Exposure to an antibiotic naturally selects for the survival of the organisms with the genes for resistance. In this way, a gene for antibiotic resistance may readily spread through an ecosystem of bacteria. Antibiotic-resistance plasmids frequently contain genes conferring resistance to several different antibiotics. This is not the case for *Mycobacterium tuberculosis*, the bacteria that causes tuberculosis,

since evidence is lacking for whether these bacteria have plasmids (ILSI, 2005). Also*M. tuberculosis* lacks the opportunity to interact with other bacteria in order to share plasmids. (ILSI, 2005;Islam *et al.*, 2001).

Genes for resistance to antibiotics, like the antibiotics themselves, are ancient. (Jayeola, 1998). However, the increasing prevalence of antibiotic-resistant bacterial infections seen in clinical practice stems from antibiotic use both within human medicine and veterinary medicine. Any use of antibiotics can increase selective pressure in a population of bacteria to allow the resistant bacteria to thrive and the susceptible bacteria to die off. As resistance towards antibiotics becomes more common, a greater need for alternative treatments arises. However, despite a push for new antibiotic therapies there has been a continued decline in the number of newly approved drugs. (Katzung and Parmely, 1989). Antibiotic resistance therefore poses a significant problem.

Although there were low levels of preexisting antibiotic-resistant bacteria before the widespread use of antibiotics (Khatune *et al.,* 2001),(Kokate, 1986) evolutionary pressure from their use has played a role in the development of multidrug resistance varieties and the spread of resistance between bacterial species (Konkwara, 1976). In medicine, the major problem of the emergence of resistant bacteria is due to misuse and overuse of antibiotics (Stephenand Kennedy,2011). In some countries, antibiotics are sold over the counter without a prescription, which also leads to the creation of resistant strains. Other practices contributing towards resistance include the addition of antibiotics to livestock feed (Lorke, 1983 and Lewington *et al.,* 1999). Household use of antibacterial in soaps and other products, although not clearly contributing to resistance, is also discouraged (as not being effective at infection control) (Mbata and Saika, 2008). Unsound practices in the

pharmaceuticalmanufacturing industry can also contribute towards the likelihood of creating antibiotic-resistant strains (Meenaa *et al*., 2009). The procedures and clinical practice during the period of drug treatment are frequently flawed — usually no steps are taken to isolate the patient to prevent re-infection or infection by a new pathogen, negating the goal of complete destruction by the end of the course (Nina *et al*., 2005).

Antibiotic resistance can be a result of horizontal gene transfer, (Obi and Onuoha, 2000) and also of unlinked point mutations in the pathogen genome at a rate of about 1 in 108 per chromosomal replication. The antibiotic action against the pathogen can be seen as an environmental pressure. Those bacteria with a mutation that allows them to survive live to reproduce. They then pass this trait to their offspring, which leads to the evolution of a fully resistant colony (Obi and Onuoha, 2000).

Antibiotic resistance can also be introduced artificially into a microorganism through laboratory protocols, sometimes used as a selectable marker to examine the mechanisms of gene transfer or to identify individuals that absorbed a piece of DNA that included the resistance gene and another gene of interest. (Ukwubile *et al.,* 2013)

The main mechanisms by which microorganisms exhibit resistance to antimicrobials are:

i. Enzymatic inactivation: The antimicrobial agent may be destroyed or inactivated by enzymes. These include β-lactamases that hydrolytically inactivate the β- lactam ring of penicillins, cephalosporins and related drugs, acetyltransferases that transfer an acetyl group to the antibacterial agent inactivating chloramphenicol or aminoglycosides, and esterases that hydrolyse the lactone ring of macrolides (Katzung, 1998; Howland and Mycek, 2006).

ii. Alteration of target site: for example, alteration of PBP—the binding target site of penicillin—in MRSA and other penicillin-resistant bacteria.

iii. Reduced drug accumulation: by decreasing drug permeability and/or increasing active efflux (pumping out) of the drugs across the cell surface. (Mbata and Saika, 2008).

iv. Alteration of metabolic pathway: for example, some sulfonamide-resistant bacteria do not require Para-aminobenzoic acid (PABA), an important precursor for the synthesis of folic acid and nucleic acids in bacteria inhibited by sulfonamides, instead, like mammalian cells, they turn to using preformed folic acid.

v. Genetic alterations:Acquired antibiotic resistance requires the temporary or permanent gain or alteration of bacterial genetic information. Resistance develops due to the ability of DNA to undergo mutation or to move from one organism to another. There may be chromosomal alteration which occurs by insertion, deletion or substitution of one or more nucleotides within the genome. The cell thus replicates and transmits its mutated properties to progeny cells. This then results in organisms that proliferate under certain selective pressures, e.g. emergence of rifampicin-resistant *Mycobacterium tuberculosis* when rifampicin is used alone. Resistance can also be acquired due to DNA transfers from bacterium to another. Resistant properties are encoded in extra chromosomal R factors (resistant plasmids). Plasmids may enter cells by transduction, transformation or bacterial conjugation (Howland and Mycek, 2006).

In fluoroquinolone resistance, mutations at key sites in DNA gyrase or topoisomerase IV can decrease their binding affinity to quinolones, decreasing the drug's effectiveness. (Nina *et al*., 2005).

2.1.3 Mechanism of Methicillin Resistance in*Staphylococcus aureus*

Although methicillin is no longer produced, the name MRSA has persisted and can be regarded as referring to resistance to virtually all β-lactam antibiotics. (Paterson *et al*., 2014).

One of the defining features of bacterial resistance is based on the physical properties of cell wall representing possible barriers for attacks by pathogens. The first form of drug resistance was ‘‘penicillin resistance’’, due to penicillin, an antibiotic of the beta lactamic family. Its resistance is due to the ability of bacteria to express beta-lactamase, an enzyme that can inactivate the drug by hydrolysis of its beta lactamic ring (Zhang *et al*., 2001 and Chambers *et al*., 1994). To overcome this phenomenon, methicillin drugs (Figure 2.2) not affected by the same molecular mechanisms of inactivation was developed with excellent results against a penicillin resistant strain because it is not a beta-lactamase substrate, although the methicillin target is the same of the penicillin.

In fact, both drugs are β-lactamic antibiotics that act by inhibiting the penicillinbinding proteins (PBPs) (Autiero *et al*., 2009) essential for cell wall biosynthesis and inhibit peptidoglycan crosslink formation, leading to bacterial cell lysis. The structure of the glycan strands of the peptidoglycan consists of repeats of a β-1, 4-linked *N*-acetyl-glucosamine-*N*acetyl-muramic acid (NAG-NAM) disaccharide (Fig. 2.3). The NAM saccharide of this pair contains a pentapeptide stem (in *S. aureus*, NAM–$_{L}$-Ala–γ-$_{D}$-Glu–$_{L}$-Lys–$_{D}$-Ala–$_{D}$-Ala), which forms a cross-link (by displacement of the terminal $_{D}$-Ala of this stem, during the transpeptidation reaction) with the residue before the first $_{D}$-Ala of the

peptide stem of an adjacent glycan chain (Fig. 2.3) (Fuda *et al.,* 2005). There are some variations among bacteria in the characteristics of the cross-linking, and in some there is an interpeptide bridge (Vollmer *et al.,* 2008). In *S. aureus* the crosslinking occurs to a pentaglycine pentapeptide extension at- tached to the ε-amine of the $_{L}$-Lys of the peptide stem of an adjacent glycan strand.

Synthesis of the glycan strand occurs in the transglycosylase domain of a biosynthetic bifunctional PBP, and stem cross-linking occurs in the transpeptidase domain of the biosynthetic PBPs. In the bifunctional PBPs, such as PBP 2 of *S. aureus* (Fuda *et al.,* 2005), the transglycosylase and transpeptidase domains are spatially well separated. Carboxypeptidases, also members of the PBP family, remove the terminal $_{D}$-Ala of the peptidoglycan peptide stems. This reaction moderates the degree of cross-linking of the peptides (Ghosh *et al*., 2008).

The methicillin- PBPs interaction causes the inactivation of the normal path to synthesize the peptidoglycan. This it does due to the fact that structural similarity of β-lactam antibiotics to the acyl- $_{D}$-Ala–$_{D}$-Ala moiety of the peptidoglycan provides the mechanistic basis for the inactivation of the PBP transpeptidase (and carboxypeptidase) domain, according to the Tipper-Strominger hypothesis (Waxman and Strominger,1983; Koch, 2003). When the β-lactam is bound in the transpeptidase/carboxypeptidase PBP domains, the identical serine nucleophile used for catalytic transpeptidation attacks the carbonyl of the β-lactam ring, resulting in serine acylation. This β-lactam-derived acyl-enzyme complex, which is stable, in contrast to the transient acyl-enzyme complex derived from $_{D}$-Ala–$_{D}$-Ala, is incapable of transpeptidation and undergoes very slow hydrolysis to regenerate the free serine.This functionally irreversible acylation stops the transpeptidase

activity of the biosynthetic PBPs and the carboxypeptidase activity of the PBPs involved in maturation of the cell wall structure.

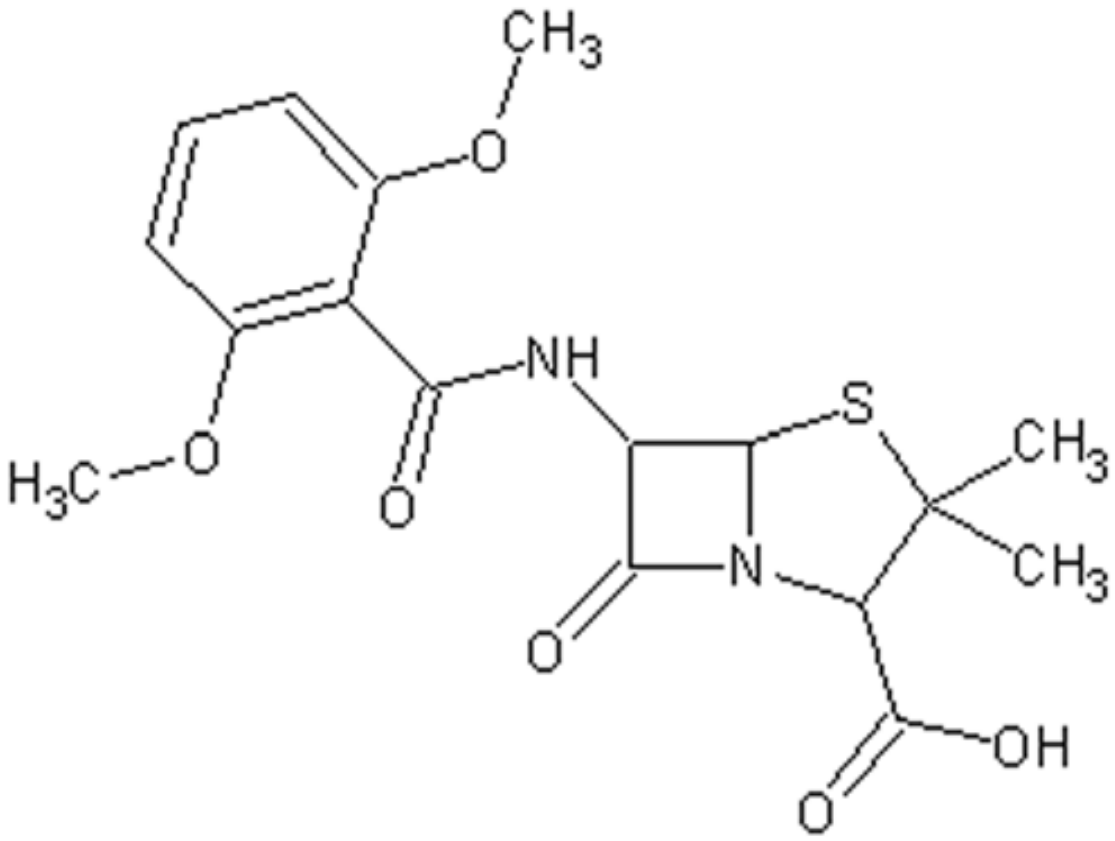


Figure 2.2:Chemical Structure of Methicillin (Adapted from Autiero *et al*., 2009).

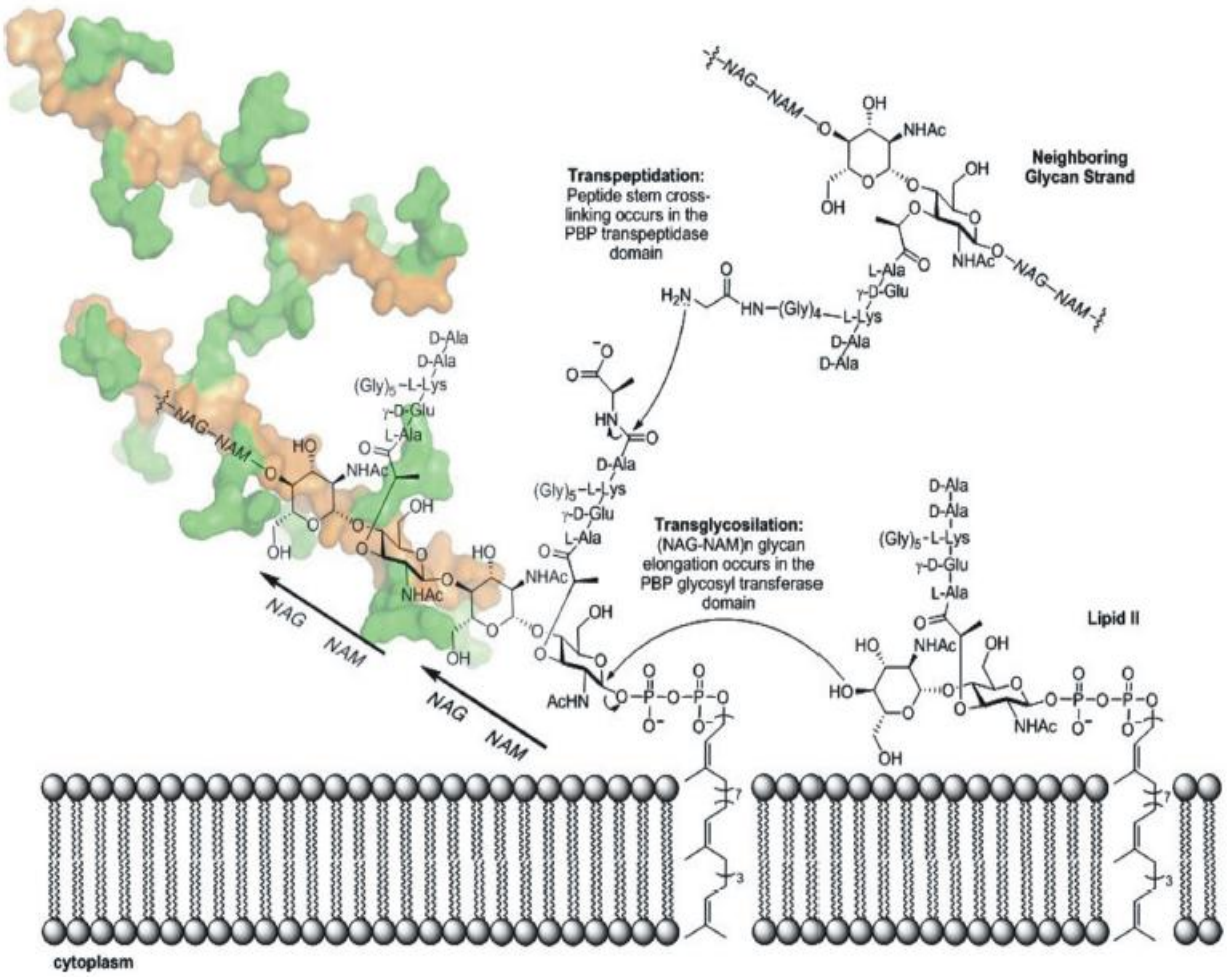


Figure 2.3: Schematic Representation of Cell Wall Synthesis in *S. aureus* (Adapted from Meroueh *et al*., 2006).

However, the basis for methicilline (β-lactam) resistance by PBP 2a-containing MRSA strainsis not due to the production of the β-lactamase enzyme, as one can see in the case ofthe penicillin-resistance (Autiero *et al*., 2009), but depends on the (PBP2a)intrinsically lower reactivity of its transpeptidase domain to β-lactam acylation (Fuda *et al*., 2004., Zapun *et al*.,2008). As PBP 2a lacks transglycosylase activity, cell wall biosynthesis in MRSA is completely dependent on the cooperative function of the PBP 2a transpeptidase with the transglycosylase domain of PBP 2 (Pinho *et al*.,2001). Hence, when the transpeptidase activity of the native staphylococcal PBPs is abolished by β-lactam acylation, the altered protein, PBP2a exhibit both a reduced rate-constant for acylation by β-lactams and elevated dissociation constants (Fuda *et al*., 2004), retaining effective transpeptidase activity while having reduced affinity for penicillin and other available β-lactam antibiotics. ThusPBP 2a functions as a surrogate to maintain cell wall synthesis at β-lactam concentrations that inhibit β-lactam-sensitive PBPs (Fuda *et al*.,2005)

In MRSA strains the resistance mechanism is based on the PBP2a expression. Moreover, some genes (mecA_GENE, mecR1_GENE and mecI_GENE,) are located on the mobile genetic elements, known as Staphylococcal Cassette Chromosomal (SCCmec) elements that constitutes 1%–2% of the ~2.9 million–bp *S. aureus* chromosome (Katayama *et al*.,2000., Hiramatsu, 2004., Robinson and Enright, 2004).SCC*mec* also contains the insertion sequence, IS431*mec,* as well as recombinases necessary for site-specific integration and excision. Some SCC*mec* types also contain various additional genetic elements, such as *Tn554* (which encodes resistance to macrolides, clindamycin, and streptogramin B), erythromycin (*erm*A and *pT18*1 (which encodes resistance to

tetracyclines) (Chambers, 2001). In particular, the mecA_GENE encodes for PBP2a when the methicillin arrives, mecR1_GENE encodes a cytoplasmic membrane-bound signal transduction receptor protein (mecR1_PROTEIN) and mecI_GENE encodes a transcriptional regulator (mecA_GENE_repressor) which is a strong repressor of the expression of the *mecA* gene. When MecR1_protein interacts with the methicillin, it breaks off, triggering a signal leading to the proteolytic release of the *mecI* repressor from the operator region of the *mecA* gene (Zhang *et al*., 2001, Archer and Bosilevac, 2001) which makes it able to repress the mecI_GENE. So, in the absence of the mecA_GENE_ soppressor, the mecA_GENE can express PBP2a (Autiero *et al*., 2009). The PBP2a expression enables the cell to synthesize the peptidoglycan and this neutralizes the methicillin effect. These PBP2a isoforms present a decrease of binding affinities for antibiotics. Therefore, PBP2a confers resistance by contributing to the function of native PBPs during cell wall synthesis (Stapleton and Taylor, 2002; Gordon and Lowy, 2008).

However, phenotypic resistance to methicillin is variably expressed, and population analysis demonstrates that each MRSA strain has a characteristic growth profile at each concentration of methicillin examined (Tomasz *et al*., 1999).

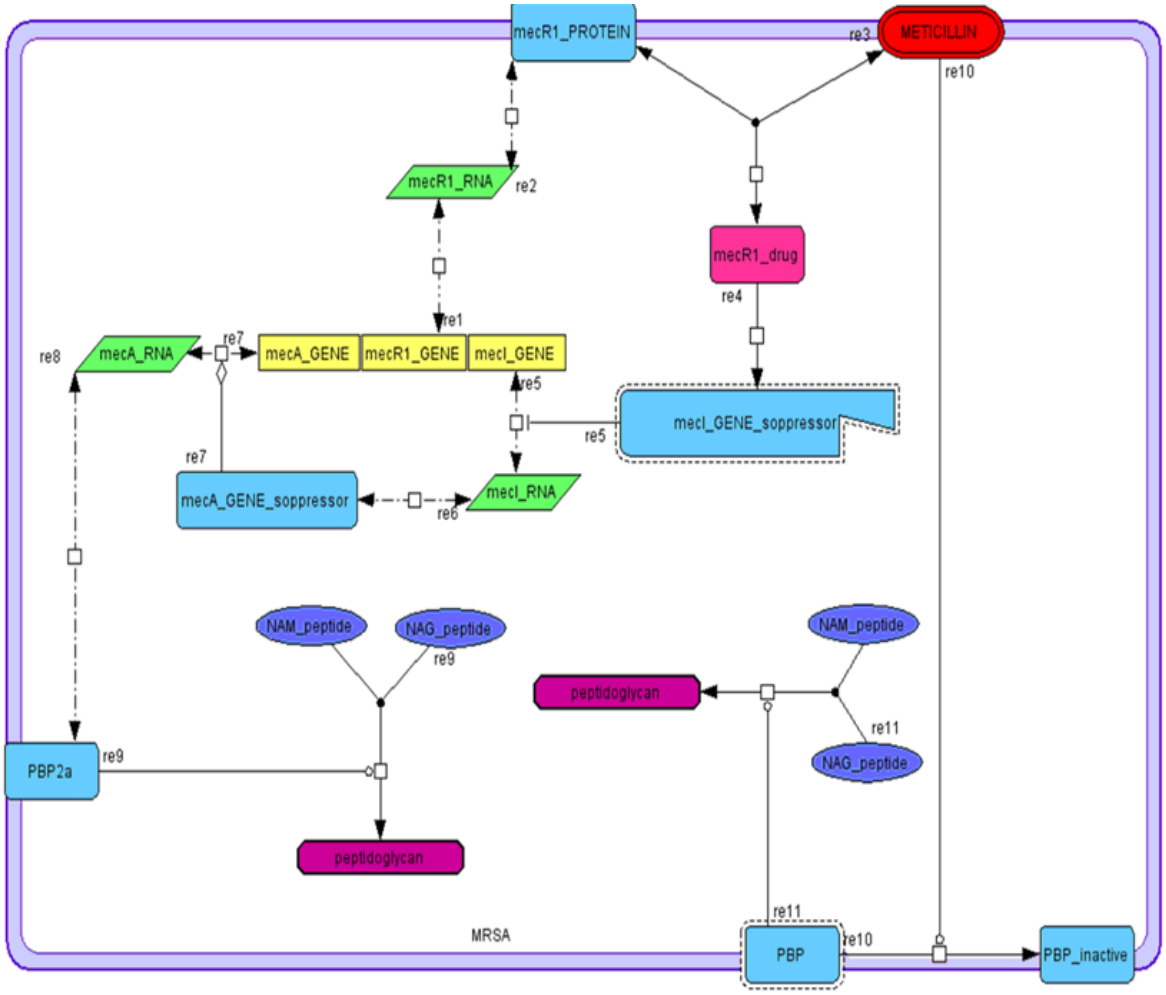


Figure 2.4:Map model Representation of The Network Connecting Genes, RNAs, Proteins and Metabolites. (Adapted from Autiero *et al*., 2009).

2.1.4Molecular Evolutionary History of MRSA

The overall genetic background of *S. aureus* isolates is unambiguously determined through multilocus sequence typing by determination of the sequence of portions of 7 housekeeping genes (Hiramatsu, 2004) onthe staphylococcal cassette chromosome mec (SCC mec).

The mobile SCC*mec* elements, on the other hand, are classified by analysis of their cassette chromosome recombinase (*ccr*) and *mecA* gene complexes (Ito *et al*., 2001) SCC*mec* types also differ with regard to their acquisition of resistance determinants acquired as the result of integration of plasmids and transposons (Ito *et al*., 2001). Like in type II and IV.At least 5 SCC*mec* types (types I–V), varying in size from ~20 kb to 68 kb, have been identified (Ito *et al*., 2004). Nevertheless, the number of SCC*mec* types has increased from type's I-VIII (Malachowa and DeLeo, 2010). The smallest of these—SCC*mec* types I, IV, and V—contain only recombinase genes and the structural and regulatory genes for resistance to methicillin and lack the transposable elements and genes encoding resistance to non–β-lactam antibiotics carried by types II and III (Ma *et al*., 2002 and Ito *et al*., 2004).

Though (year 2010) study has shown that the smallest of these –SCC*mec* types are now types I, IV, V, VI in which I, V and VI contain only recombinase genes and the structural and regulatory genes for resistance to methicillin and lack the transposable elements and genes encoding resistance to non–β-lactam antibiotics carried by types while IV has the same characteristics but with additional aminoglcoside resistance due to Tn*4001* encoding an aminoglycoside resistance operon (*aacA-aphD*). SCC*mec* types I-IV contain alleles of *ccrA* and *ccrB,* whereas type V,which has to date been identified in a small number of AustralianCA-MRSA isolates, contains a novel *ccr* designated *ccrC* (Ito *et al*., 2004).

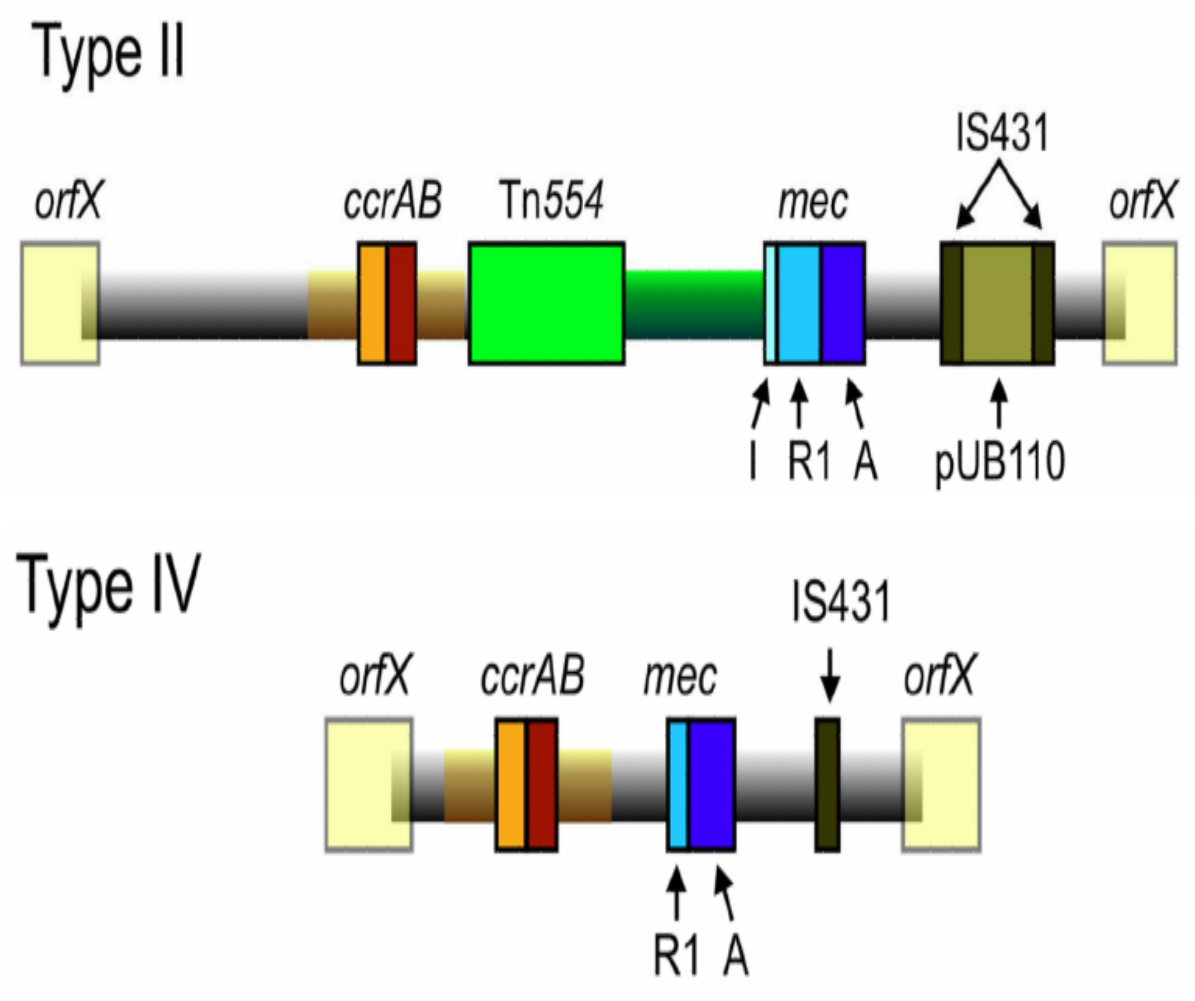


Figure 2.5: Comparison of Methicillin-Resistance Cassettes Typical of Hospital- or Community MRSA (Adopted from Chambers and DeLeo, 2010).

Though (year 2010) study has shown futher that it's now SCC*mec* type I, II, III, IV, VI and VIII contain alleles of *ccrA* and *ccrB,* while SCC*mec* type V and VII contains the novel *ccrC*.

A novel resistance gene mecC MRSA has been discovered, validating the report of methicillin-resistant Staphylococcus aureus (MRSA) encoding a divergent *mec*A gene in 2011 (Paterson *et al*., 2014). This homologue, designated *mecC*, poses diagnostic problems with the potential to be misdiagnosed as methicillin-sensitive S. aureus, with important potential consequences for individual patients and for the surveillance of MRSA. *mecC* MRSA have now been reported from 13 European countries and have been isolated from 14 different host species, with evidence of a recent increase in Denmark. (Paterson *et al*., 2014). *mecC* gene was formally called mecALGA251 because it is of a *S. aureus* isolate, LGA251, during 2007 epidemiological study of bovine mastitis (García Álvarez, *et al.*,2011[a])from a bulk tank milk sample in southwest England which was phenotypically MRSA (i.e., resistant to oxacillin and cefoxitin). However, confirmatory tests for the mecA gene and PBP 2a/2' were repeatedly negative (García-Álvarez *et al*., 2011[b]). Genome sequencing of LGA251 at the Wellcome Trust Sanger Institute revealed that the strain carried a novel *mec*A homologue, initially termed *mec*A_{LGA251}, which was only ~69% identical to conventional *mec*A at the DNA level , and the encoded PBP 2a/2' was ~63% identical at the amino acid level (García-Álvarez *et al*., 2011[b]). This explained the resistance of LGA251 and why it produced negative results by *mec*A PCR and PBP 2a/2' slide agglutination. A retrospective search of isolate collections in the UK and Denmark identified a further 65 isolates positive for *mec*A_{LGA251} isolated not only from dairy cattle

but also from humans, including the earliest known isolate, a Danish blood isolate from 1975 (García-Álvarez *et al*., 2011) [b]. In consequence, although *mec*A$_{LGA251}$ MRSA has only recently been recognised, it may have been causing human infections for over 35 years. These *mec*A$_{LGA251}$ MRSA isolates belonged predominantly to CC130 and ST425 (García-Álvarez *et al*., 2011[b]). Similarly to conventional *mecA*, *mec*A$_{LGA251}$ is located within a SCC*mec* element inserted into the 3' region of *orfX*.

The LGA251 SCC*mec* was also novel; it had divergent *ccrA* and *ccrB* recombinases (belonging to the *ccrA1* and *ccrB3* groups and representing a novel combination of recombinase groups designated type 8 *ccr*), divergent *mecA* regulatory genes (*mecI/mecR*), and the absence of one of the three joining regions (J3) that are normally present (García-Álvarez *et al*., 2011[b]). The SCC*mec* sequence from LGA251 was submitted to the Working Group on the Classification of SCC and given the designation type XI SCCmec in November 2009. *mecA*$_{LGA251}$ was itself subsequently renamed *mec*C in 2012 (Ito*et al*., 2012). *mec*C was chosen because an additional divergent homologue of mecA, distinct from *mecA*$_{LGA251}$, had already been described in *Macrococcus caseolyticus* (Tsubakishita*et al*., 2010)and was designated *mecB*(Ito*et al*., 2012). Published at the same time as the UK and Danish report (García-Álvarez *et al*., 2011[b]), work in the Republic of Ireland independently described *mec*C and type XI SCC*mec* in human MRSA strains isolated in 2010 and belonging to CC130 (Shore *et al*.,2011).

Genetic evolutionary analyses have demonstrated that the *mecA* gene has been transferred into methicillin-susceptible *S.aureus* (MSSA) on ⩾20 occasions, having emerged in ⩾5 phylogenetically distinct lineages (as well as reemerging within individual lineages) (Enright *et al*., 2000, Enright *et al*., 2002and Hiramatsu, 2004,). It has been suggested that

the emergence of PBP2a initially resulted from a recombination event involving the genes encoding an existing PBP and an inducible β-lactamase (Song *et al*., 1987). The donor strains that became the source of PBP2a are likely to have been coagulase-negative staphylococci, with *Staphylococcus sciuri* identified as a prime candidate (Wu *et al*., 2001). A recent study of 44 methicillin-resistant *Staphylococcusepidermidis* isolates from the blood of patients with prosthetic valve endocarditis from 1973 to 1983 found that 2% carried SCC*mec* type I, 34% carried type II, 28% carried type III, and 36% carried type IV (Wisplinghoff *et al*., 2003). The introduction of *mecA* from the putative donor species into MSSA strains that are already successfully adapted to hospital environments and to the community have, in turn, created successful epidemic HAMRSA and CA-MRSA clones (Enright*et al*., 2000., Ma *et al*., 2002., Okuma *et al*., 2002., Baba *et al*., 2002).

Evidence indicates that the ancestral MRSA genotype, ST250- MRSA, is a strain originating in Denmark and possessing SCC*mec* type I, most extant isolates of which were obtained in the 1960s (Enright *et al*., 2002). (By convention, strains are named by their sequence type [ST] and the presence or absence of methicillin resistance. Thus, this strain is a methicillin-resistant *S. aureus* of a sequence type designated as 250). ST250-MRSA arose as the consequence of the acquisition of the mec gene by the methicillin-susceptible strain ST250-MSSA, which had itselfrisen from ST8-MSSA by a chromosomal point mutation (Enright *et al*., 2002). ST250-MRSA is no longer a major cause of epidemic MRSA infections, but ST247-MRSA (the "Iberian clone"), which evolved from ST250-MRSA by a single point mutation, remains an important hospital pathogen in Europe and has been reported to have caused an outbreak in a New York City hospital (Roberts *et al*.,1998). As indicated above, there have since been multiple introductions of *mec* into *S.*

aureus (Enright *et al.*, 2000). The emergence of CAMRSA strains, in particular, has repeatedly occurred as a result of the introduction of SCC*mec* type IV into a variety of genetic MSSA backgrounds (Okuma *et al*., 2002). In the United States, one of the resultant clones, ST8-MSSA (USA 300) has proven increasingly successful (Perdreau-Remington *et al*., 2004).

2.1.5 Functional Characterisation of *mec*C-encoded PBP2a

The function of the *mecC*-encoded PBP 2a/2' and its role in β-lactam resistance was formally demonstrated by the work of Kim *et al*. which also highlighted noteworthy differences in the properties of the *mecA* and *mecC*-encoded proteins (Kim *et al*., 2012). Although the detection of *mecC*-encoded PBP2a in LGA251 was problematic, most likely due to low expression levels resulting from *mecI/mecR,* inducible expression of *mecC* in a methicillin-sensitive *S. aureus* (MSSA) strain conferred high minimum inhibitory concentration (MIC) values against a range of β-lactams (Kim *et al*., 2012). Recombinant PBP2amecC protein was bound by β-lactams but showed higher affinity for oxacillin compared to cefoxitin, whereas PBP2amecA showed less preference. The two proteins also displayed differences in their thermostabilty and temperature optima, with PBP2a*mecC* appearing to be less stable at 37 ^{o}C than PBP2a*mecA*. Interestingly, PBP2a-*mecC* did not require the presence of the native PBP2 to confer high-level oxacillin resistance. This is in contrast to PBP2amecA for which high-level oxacillin resistancerequires the presence of native PBP2 to provide transglycosylase activity lacking in PBP2a*mecA*. Because PBP2a-*mecC*also appears to lack transglycosylase activity, highlevel oxacillin resistance conferred by *mecC* is likely to involve collaboration between PBP2a*mecC* and one of the other monofunctional glycotransferases that are known to be induced in *S. aureus* when PBP2 is

inhibited (Kim *et al*., 2012). Although this characterisation confirms the function of *mecC*-encoded PBP2a as a transpeptidase, and its role in methicillin resistance, there are important differences in the behaviour of the proteins encoded by *mecC* and *mecA*. The structural and evolutionary bases for these distinctions are not yet clear (Paterson *et al*., 2014).

2.1.6 Resistance to Antibiotics Other Than β-Lactams

In contrast to the multidrug resistance usually seen in HAMRSAstrains, antibiotic resistance in CA-MRSA strains is often limited to β-lactams. The small size of SCC*mec* type IV maypreclude its carriage of additional genetic material, in contrastto the characteristic presence of additional genetic material inSCC*mec* type II and SCC*mec* type III (Hiramatsu,2004;Robinson and Enright, 2004). This does not,however, preclude chromosomally encoded resistance or thepresence of resistance plasmids in strains carrying any of the*mec* types. For instance, some CA-MRSA strains isolated inWestern Australia contain a 41.4-kb plasmid encoding resistanceto tetracycline and trimethoprim, as well as resistance to mupirocinand cadmium (Pearman and Grubb1993&Udo*etal*., 1994). Fluoroquinolone resistance isfrequent in CA-MRSA carrying SCC*mec* type IV isolated fromhomeless youth in San Francisco (Pan*et al*., 2003). Nonetheless, in contrastto HA-MRSA strains, most CA-MRSA isolates remain susceptibleto tetracyclines, clindamycin, and trimethoprim-sulfamethoxazole (TMP-SMZ) (Naimi *et al*.,2003).

2.1.7 Composition of the Gene Cassette Coding For Methicillin Resistance

Epidemiologic success and virulence of CA-MRSA strains differ in a number of important ways fromthe 6 major pandemic clones of MRSA that account for nearly70% of epidemic HA-MRSA strains (Aires de Sousa and de Lencastre, 2003). These differences arefound in

the composition of the gene cassette coding for methicillinresistance, in the carriage of plasmids encoding resistance to antibiotics of other classes (as well as resistance to heavy metals), and in their associated virulence factors.

The earliest strain of MRSA in which SCC*mec* type IV has been identified was isolated in 1981 (Ito *et al*., 2001). Despite this apparently recent emergence, an analysis of a large number of MRSA isolates detected SCC*mec* type IV in twice as many clones as any of the other types, suggesting its greater promiscuity and successful persistence (Robinson and Enright, 2004)**.** This may be the result of greater efficiency of transfer and or a lesser fitness cost to the recipient clone, possibly because of its smaller size and lack of the "excess baggage" included in other SCC*mec* types (Ma *et al.*,2002, Okuma*et al*., 2002,Robinson and Enright2004). Although HA-MRSA has been reported to replicate more slowly than MSSA (Sutherland andRolinson, 1964), a CA-MRSA clinical isolate harboring SCC*mec* type IV has been demonstrated to replicate more rapidly than HAMRSA isolates with other SCC*mec* types (Okuma*et al*., 2002 and, Baba*et al*., 2002). In contrast, transformation of a SCC*mec* type I element into *S. aureus* strains yielded highly oxacillin-resistant transformants with a reduced growth rate (Ender*et al*., 2004). This relatively greater fitness of CAMRSA strains carrying SCC*mec* type IV may account for its remarkable success in displacing other MRSA strains in some hospitals after its introduction from the community (Donnio *et al.*, 2004).

2.1.8Detection and Diagnosis of MRSA

The detection and diagnosis of MRSA in the clinical microbiology setting is very important both for informing the appropriate treatment of individual patients and also for the surveillance of MRSA. The gold standard for confirmationof MRSA is regarded as the

molecular detection of either *mecA*, typically by PCR, or of PBP2a/PBP20, usually by antibody detection with commercially available slide agglutination assays. Crucial to the reliability of these assays is the fact that *mecA* and PBP2a/PBP20 are both highly conserved among MRSA isolates (Paterson *et al*., 2014).

2.2 History of antimicrobial agents

Flemming noted the inhibition of bacteria by a colony of *Penicillum notatum* that had developed as a contaminant on a Petri dish (Howland and Mycek, 2006).

By 1940, significant quantities of the first penicillin from cultures of *Penicillum notatum* were produced. Penicillin G had clinical limitations which were its instability at acidic pH, susceptibility to destruction by beta-lactamase (penicillinase) and its relative inactivity against Gram-negative bacteria. These limitations were overcome by the production of semi-synthetic penicillins (Katzung, 1998).

Resources of industry and academic institutions were devoted to the study of penicillins and search for other antibiotics leading to the discovery of streptomycin, aureomycin, chloromycetin and other antibiotics involving various species of *Streptomyces, Cephalosporium* and *Penicillum* (Kong and Liang, 2003; Howland and Mycek, 2006)

Unfortunately, the widespread and indiscriminate use of antibiotics, together with poor hygiene has led to many pathogenic organisms acquiring resistance to specific antibiotic treatments.

This problem is worsened by the fact that resistance to a particular antibiotic can be transferred from one organism to another. Thus, the clinician's antibiotic armamentarium is being steadily eroded, increasing the need for research in isolation or synthesis of new drugs (Trease andEvans, 2002).

2.2.1 Conventional antimicrobial agents

Antimicrobial agents can be categorised according to whether they are antibiotics (derived from the growth of microorganisms), chemotherapeutic agents (synthetic compounds not found in nature) or derivatives from nonmicrobial natural sources (lichens, higher plants and animals) (Trease and Evans, 2002). According to Walkman's 1951 definition of antibiotics, the term is limited to substances produced by microorganisms. The term antibacterial is consequently used to include those active compounds prepared synthetically or isolated from higher plants. Most of the clinically used antibiotics are produced by soil microorganisms or fungi (Trease and Evans, 2002).

Antibacterial drugs are classified as either bacteriostatic or bactericidal. Bacteriostatic drugs arrest the growth and replication of bacteria at serum levels achievable in the patient, thus limiting the spread of infection while the body's immune system attacks, immobilises and eliminates the pathogens. If the drug is removed before the immune system has scavenged the organisms; enough viable organisms may remain to begin a second cycle of infection. Bactericidal drugs kill bacteria at drug serum levels achievable in the patient. Because of their more aggressive antimicrobial action, these agents are often the drugs of choice in seriously ill patients (Howland and Mycek, 2006).

The antibacterial drugs may be divided by the spectra of bacteria for which they are therapeutically effective i.e. narrow-spectrum, extended spectrum and broad spectrum:

(i) Narrow-spectrum antibacterial drugs are those that act only on a single or limited group of microorganisms. For example isoniazid which is active only against mycobacteria.

(ii) Extended spectrum antibacterial drugs are those that are effective against Gram-positive organisms and also against a significant number of Gram-negative bacteria, e.g. ampicillin which acts against Gram-positive and some Gram-negative bacteria.

(iii) Broad-spectrum antibacterial drugs are exemplified by tetracycline and chloramphenicol which affect a wide variety of microbial species (Howland and Mycek, 2006).

2.3 Medicinal Plants

Medicinal plants are a significant aspect of developing a safer antibacterial principle through isolation, characterization, identification and biological studies (Chanda *et al*.,2010), and despite the availability of different approaches for the discovery of therapeuticals, natural products, of course from medicinal plants still remain one of the best reservoirs of structural types (Hostettmann, 1999). The use of medicinal plants as a source for relief from illness can be tracked back over five millennia to written documents of the early civilization in China, India and near east of Asia, and it is doubtless an art as old as mankind (Prozesky *et al*., 2001) Nevertheless we can say that the use of medicinal plants all over the world predates the introduction of antibiotics and other modern drugs into Africa continent (Akinyemi *et al.*,2005).

In Africa, The practice of herbal medicine which employs plants as its major components is an integral part of traditional and culture of Africans (Mann *et al.,* 2003). Many medicinal plants are used traditionally in most African countries, with the traditions of collecting, processing and applying plants and plant based medications having been handed down orally from generation to generation (Von Maydell, 1996).

The Nigerian environment is probably the least explored in terms of available untapped resources. Nigeria flora has over seven thousand, three hundred and forty-nine species of higher plants that had made serious impact on the health and wealth of Nigerians and could be an enormous source of foreign exchange for the country (Mann *et al*., 2003).

Many medicinal plants have been studied scientifically and in most cases the results confirmthe traditional therapeutic claims of these plants (Samy *et al*., 1998). For example the root bark of *T. avicennioides* from many research worksindicates the presence of anthraquinone, saponins, steroids, tannins terpenes flavonoids and triterpenoids which possess anti-inflammatory properties; traditionally, the root bark is used by the locals and is considered cleansing and healing on refractory sores (Kerharo and Adam, 1963).

Medicinal plants have therapeutic properties due to biosynthesis of various complex phytochemical substances grouped broadly as phenolics, alkaloids and terpenoids. Synergistic interaction among the multiple phytochemicals may be responsible for the overall bioactivity of a given medicinal plant. Pharmacological and clinical studies of phytochemical in plants have shown that they exhibit various medicinal uses and serve as the major backbone of traditional medicine (Van Wyk and Wink, 2004).

2.3.1*Terminaliaavicennioides,* Taxonomy and Uses

Terminalia avicennioides belongs to the family Combretaceae, genus *Terminalia*, and species *avicennioides.* It is a yellowish brown, hard and durable wood; grow as shrubs or small trees (Burkill, 1985) commonly found in the Savannah region in West Africa (Mann *et al*., 2011), woodland from Senegal to N and S Nigeria, and on into Cameroun and Ubangi-Shari. The rough grey corky bark renders the tree somewhat fire-resistant. In

Nigeria, it is called baúsheè in Hausa,bárbár, in Kanuri,kpace in Nupe, kuegh inTiv,idin inYorubaand edo in Igbo. Itis a yellowish brown, hard and durable wood. The Hausa of N Nigeria make bows and walking-sticks or cudgels from the roots, which are first wrapped in leaves and scorched to remove the bark, and then soaked in oil. Any staff or cudgel called gwalmi or kwaleru (Hausa) is usually said to be made of wood of this species. in NE Nigeria the Jukun use the roots in a ceremonial treatment of syphilis (Irvine, 1961). The pulverized leaves are used in N Nigeria on burns and bruises. The leaves are often galled, called in N Nigeria galulai-baushi (Hausa), and these have medicinal use: reduced to ashes and mixed with roasted bulbs of Crinum (Amaryllidaceae), an ointment is prepared with fresh cow's butter for rubbing on rheumatic and swollen joints(Burkill, 1985).

Traditionally, the aqueous extract of its stem bark is employed to treat sleeping sickness (Atawodi *et al.*, 2002). Extracts of the stem bark and rootsare used for the treatment of bacterial infections such as diarrhea and gastrointestinal disorders in Nigeria (Abdullahi *et al*., 2001; Irvine, 1961), dental caries and skin infections (Lewis and Elvin-Lewis, 1977); bloody sputum (tuberculosis) and cough in humans (Mann *et al*., 2007a), The roots which are used as chewing sticks have been claimed to cure dental caries and skin infections (Gill and Akinwunmi, 1986).

Pharmacologically, crude methanolic extract of the *T. avicennioides* root bark exhibited broad growth inhibition against microbes causing infectious diseases (Mann *etal*., 2007b; 2008c) and it was found to significantly inhibit activity of *Mycobacterium tuberculosis* (Mann *et al*., 2008b). Previous studies, showed that the stem bark extract of *T. avicennioides* exhibited both vibrocidal and typhoidal activities (Akinyemi *et al*., 2005), antimicrobial activity (Mann et al., 2008a) and malaria parasite (Sanon *et al.*,2013). Its

efficacy on the healing of ulcer and wound has been also reported (Akinyemi *et al*., 2005). Also methanol extract of this plant suggested potent anti-trypanosomal activity (Atawodi *et al.*, 2003).

Terpenoids has been detected phytochemically in an inbitory fraction of *Terminalia avicennioides* Guill and Perr (Root), assayed against *Staphylococcus aureus,* (Mann*et al.*,2009). However, the study was limited to *Staphylococcus aureus* and not extended to Methicillin Resistant *Staphylococcus aureus* as the later has a modified genetic makeup conferring resistance, thus bringing possible differences amongst the two in their activity with the plant. Also the study was done with root and not the root bark. Nevertheless,characterization for the specific compound(s) associated with this very activity was not identified, since terpenoids are large and diverse naturally occurring organic chemical which differ from each other not only in functional groups but also in their basic carbon skeleton.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant material

*Terminalia avicennioides*leafandroot bark were collected from their natural habitat in its natural habitatin Samaru, Zaria L.G.A Kaduna State. The plant materialswere identified and authenticated at the Herbarium in the Department of Biological Science, Ahmadu Bello University Zaria, with the voucher number 901452.

3.1.2 Chemicals and Reagents.

Folin's reagent (Hopkins and Williams, England)., Glacial Acetic acid (Avondale Laboratories Ltd Banbury, Oxon, England), Muller Hinton broth, Muller Hinton agar (Oxoid, Basingstoke-Hampshire, UK), all other chemicals: and all other reagents: Normal Saline, n-Hexane, Chloroform, Ethyl acetate, Methanol, Dimethyl sulfoxide, were of analytical grade.

3.1.3 Equipments used

UV-visible spectrophotometer, GC-MS machine (QP 2010 plus Shimadzu, Japan), FTIR UV-spectrophotometer 1800(Shimadzu model), electronic weighing balance, incubator, Refrigerator, Soxlet extractor, Oven, bunsen burner, micropipette, quartz curvette, syringes (1 ml, 5ml, 10ml), ultracentrifuge and water bath were used.

3.1.4 Bacterial Isolate

ATCC 33591 standard strains of methicilin resistant *Staphylococcus aureus*(University Boulevard Manassas VA 20110 USA)obtained from Department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University Zaria was used.Resistance of

Figure 3.1: *Terminalia avicennioides* Plant in its Natural Habitat inSamaru, Zaria L.G.A, Kaduna State.

this strain to Methicillin/Oxacillin antibiotic was verified by a retest of its sensitivity to Oxacillin antibiotic(Oxoid, UK)and interpreted as described in the performance standard

for antimicrobial susceptibility testing as described in the Cinical and Labouratory Standard Institute 2007. Picture is shown in Appendix 1.

3.2 METHODS

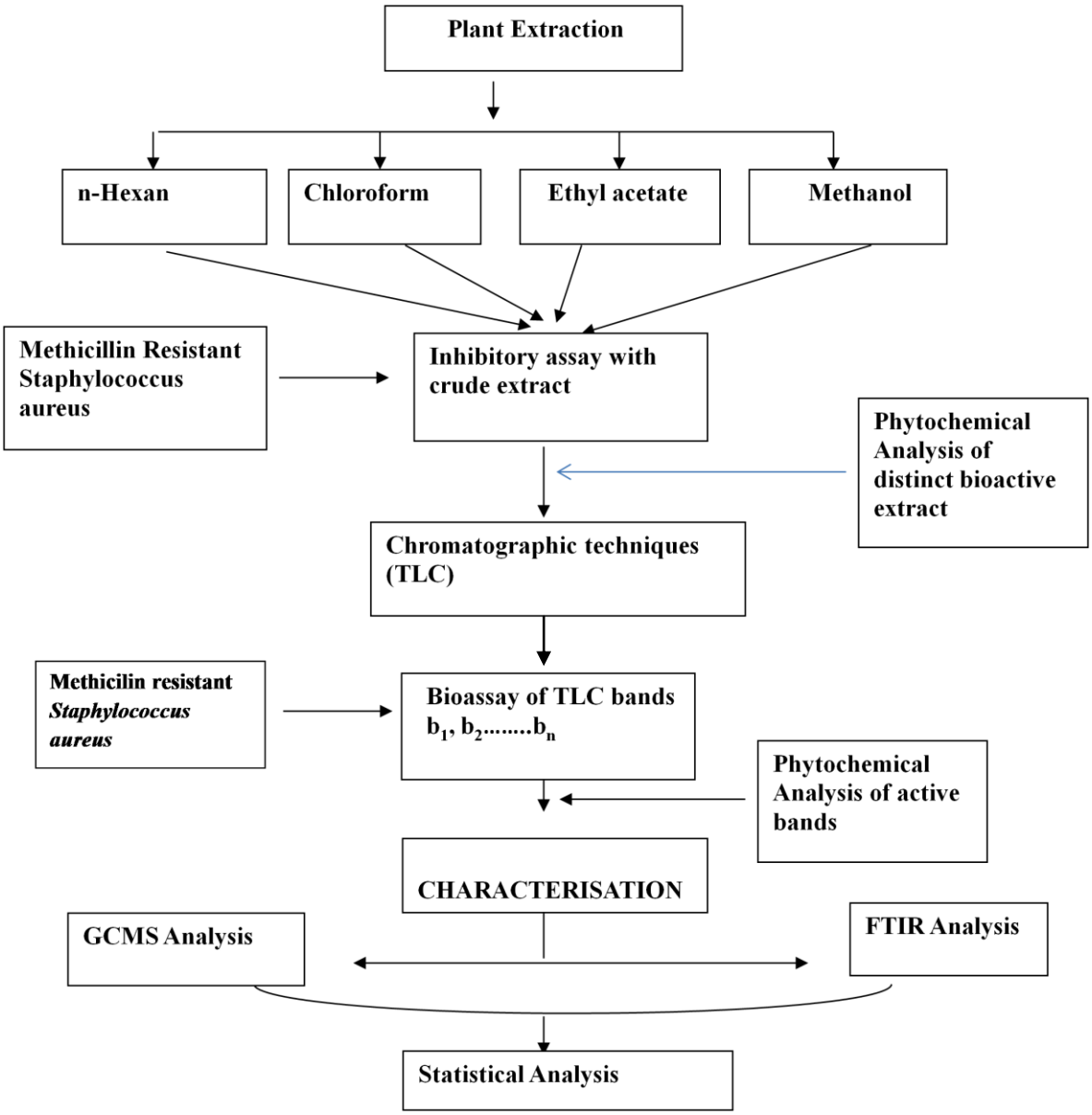


Figure 3.2Exeprimental Design

3.2.1 Preparation of Plant Materials

The leaf and root bark were separated washed and dried at room temperature for 14 days.The dried plant parts were then pulverized using pestle and mortarsieved and stored in polythene bag until required.

3.2.2 Preparation of Plant Extracts

Three hundred grams (300g) each of the powdered *Terminalia avicennioides* leaf and root barkwere extracted successively by cool maceration with 750ml, n-Hexane, Chloroform, Ethyl acetate and Methanol, independently for 48hrs using soxhlet extractor. The extracts were filtered using whatmanfilter paper No.1and left at open air to dry at room temperature. The percentage yield of each extract was determined by the formula:

$$\frac{\text{Mass yield of Extract}}{\text{Mass of Plant Material}} \times \frac{100}{1}$$

3.2.3 Antimicrobial Activity (Bioassay) of Crude Extracts

The antimicrobial test was performed using the agar well diffusion method of (Nair *et al.*, 2005). A 0.1ml standardized inoculum (1.5×10^8 cfu/ml 0.5 McFarland standards) of a 24hrs culture was inoculated or spread using a sterile cotton swab onto the surface of sterile Muller Hinton agar which has been prepared and allowed to solidify. The excess of the inoculums was allowed to drain off. The plates were left on the bench for 1hour for the inocula to diffuse into agar. Wells of 5 mm diameter were made on the agar using a sterile cork borer. The cut agar disks were carefully removed by the use of forceps sterilized by flaming. To each well was introduced a 0.5 ml of each extract graded concentrations (60µg/ml, 120µg/ml, 180µg/ml, and 240µg/ml) of each of the solvent crude plant extracts dissolved in Dimethylsulfoxide (DMSO) respectively.

Inoculated plates with wells to which DMSO instead of plant extract has been added was used as negative controls while standard antibacterial drug (Aurgumentin) was used as

positive control.The plates were inoculated at 37^0C for 24 hrs. The zones of inhibition (clearance) around the agar well and standard drug were measured in (mm) from the edge of the plate to the point where the growth of the organism started after incubation and compared with that in Kirby Bauer chart.

3.2.4 Qualitative Phytochemical Screening:

Phytochemical screening of alkaloid, flavonoids, tannins, cardiac glycosides, terpenoids anthraquinones, of the distinct bioactive crude extractof *Terminaliaavicennioides*on methicillin resistant *Staphylococcus aureus* was conducted following the method of Harbone, (1998) and Trease and Evans, (1989)., but with a 10 percent scale down of plant extract and reagents.

3.2.4.1Test for Alkaloids

The extracts (0.5 g) was diluted in 10 ml of 1% aqueous hydrochloric acid, boiled and filtered.Dilute ammonia 2 milliliter (ml) was added to 5 milliliter (ml) of the filtrate. Chloroform 5 milliliter (ml) was added and shaken gently to extract the alkaloidal base. The chloroform layer was extracted with 10 ml of acetic acid. This was then divided into two portions. Mayer's reagent was added to one portion and Draggendorff's reagent to the other. The formation of a cream (with Mayer's reagent) or reddish brown precipitate (with Draggendorff's reagent) was observed indicating the presence of alkaloids.

3.2.4.2 Test for Flavonoids

Two methods were used to test for flavonoids. First, 10% of dilute ammonia (5 ml) wasadded to a portion of an aqueous filtrate of the extracts. Concentrated sulphuric acid (1 ml) was added. A yellow colouration that disappeared on standing indicated the presence of flavonoids. Second, a few drops of 1% aluminium solution was added to a portion of the filtrate. A yellow colouration indicated the presence of flavonoids.

3.2.4.3 Test for Tannins

The extract (0.5g)was boiled in 10 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish-green or a blue-black colouration, indicating the presence of tannin.

3.2.4.4 Test for Cardiac Glycosides (Keller-Killiani test)

To 0.5 g of the extract diluted in 5 ml of water, 2 ml of glacial acetic acid containing one drop of ferric chloride solution was added. concentrated sulphuric acid1 ml was added. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring appeared below the brown ring, while in the acetic acid layer a greenish ring formed just above the brown ring and gradually spread throughout the layer.

3.2.4.5Test for Triterpenes (Lieberman Buchard test)

To 0.5ml of sample was added 5ml of acetic anhydrideand shaken, then 1ml of $H_2SO_4$was added. Formation of a brown ring at the interface indicates the presence of triterpenes.

3.2.4.6 Test for Terpenoids (Salkowski test)

To 0.5 g of each of the extracts, 2 ml of chloroform was added. 3 ml of concentrated sulphuric acid (H_2SO_4) was as well carefully added to form a layer. A reddish brown colouration of the interface indicated the presence of terpenoids.

3.2.4.7Test for Anthraquinones

The extract(0.5g) was boiled with 10 ml of sulphuric acid (H_2SO_4) and filtered using Whatman filter paper No.1. The filtrate was shaken with 5 ml of chloroform. The chloroform layer was pipetted into another test tube and 1 ml of 10% of dilute ammonia was added. The resulting solution was then observed for colour changes.

3.2.5 Quantitative Phytochemical Analysis

3.2.5.1QuantitativeEstimation of Alkaloids

This was done using a spectrophotometric method precipitable as described by Sreevidya and Mehrotra, (2003). It is based on the formation of yellow bismuth complex in nitric acid medium with thiourea.Plant material (10 mg) was dissolved in 10ml methanol and termed a Stock solution. A 5 ml amount of the stock solution was taken and thepH was maintained at 2–2.5 with dilute HCl. A 2 ml amountof DR was added to it, and the precipitate formed was centrifuged.The centrifugate was checked for complete precipitationby adding DR. After centrifugation, the centrifugate wasdecanted completely and meticulously. The precipitate wasfurther washed with alcohol. The filtrate was discarded andthe residue was then treated with 2 ml disodium sulfide solution.The brownish black precipitate formed was then centrifuged.Completion of precipitation was checked by adding 2drops of disodium sulfide. The residue was dissolved in 2 mlconcentrated nitric acid, with warming if necessary. This solutionwas diluted to 10ml in a standard flask with distilled water;1 ml was then pipetted out, and 5 ml thiourea solutionwas added to it. The absorbance was

measured at 435 nm against the blank containing nitric acid and thiourea. The amount of bismuth present in the solution was calculated by multiplying the absorbance values with the factor, taking suitable dilution factor into consideration. The factor is obtained from the standard curve, which is a constant for different concentrations.

Factor = concentration/absorbance

Because the Bismuth Nitrate-Thiourea complex formed is 1:1, the amount of bismuth corresponds to the amount of alkaloids present; hence the method of preparation of DR does not affect the result. The amount of alkaloid present is calculated by multiplying absorbance with factor.

3.2.5.1.1 Procedure for Calibration Curve

The calibration curve was obtained with bismuth nitrate pentahydrate stock solution. Serial dilutions of the stock solution were made by pipetting out 1, 2, 3, 4, 5, 6, 7, 8, and 9 ml stock solution into separate 10ml standard flasks and diluting to volume with distilled water. A1 ml amount of this solution was taken, and 5 ml thiourea solution was added to it. The absorbance value of the yellow solution was measured at 435 nm against colorless reagent blanks. Alkaloids concentration was calculated from regression equation of the standard curve of bismuth nitrate–thiourea complex{Bi [CS $(NH_2)_3$]} $(NO_3)_3$}. The preparation of all standard reagents for quantitative estimation of alkaloids are presented in Appendix 2.0

3.2.5.2 Quantitative Estimation of Tannins

This was done with a slight modification of method of Swain *et al*.,(1979)as follows. finely ground sample 20mgwas measured into a 5 ml beaker. 2 ml of 50% methanol was added and covered with paraffin and placed in a water bath at 80^oC for 1 hr and stirred with a

glass rod to prevent lumping. The extract was quantitatively filtered using a double layered Whatman filter paper No.1 into a 10 ml volumetric flask using 50% methanol to rinse. This was made up to mark with distilled water and thoroughly mixed. Sample extract (0.1 ml) was pipetted into 5 ml volumetric flask, 2 ml distilled water, 0.25 ml Folin-Denis reagent and 1 ml of 17% $Na_2CO_3$was added and mixed properly (preparation of 17% $Na_2CO_3$is presented in Appendix 2.0). The mixture was made up to mark with distilled water, mixed well and allowed to stand for 20 min when a bluish-green colouration developed. Standard tannic acid solutions of range 0-10 ppm were treated similarly as 1 ml of sample above. The absorbances of the tannic acid standard solutions as well as samples were read after colour development on a spectrophotometer at a wavelength of 760 nm. Percentage tannin was calculated from regression equation of the standard curve of tannic acid.

3.2.5.3 Quantitative Estimation of Anthraquinone:

This was done with a slight modification of method described by Soladoye and Chukwuma(2012) as follows.Fine powder sample (10 mg) was soaked in 10 ml of distilled water for 16 hours. This suspension was heated in water bath at 70^{o}C for one hour. After the suspension was cooled, 10ml of 50% methanol was added to it and then filtered. The clear solution was measured by spectrophotometer at a wavelength of 450nm and compared with a standard solution containing 1mg/100ml alizarin with the absorption-maximum 450nm. Calculation for antraquinon was done using

$$\frac{C_I}{C_2A_2} = \frac{A_1}{}$$

Where C_1 = Concentration of Sample.

C_2 = Concentration of Standard.

A_1 = Absorbance of Sample.

A_2 = Absorbance of Standard.

3.2.6 Fractionation of The Most Bioactive Crude Extract of *Terminalia avicennioides* By Preparative Thin Layer Chromatography

Preparative Thin Layer Chromatography (PTLC) was carried out on the ethyl acetate root bark extract of *Terminalia avicennioides* using stationary phase: Silica gel 60 F_{254} pre-coated TLC plates by Merck of 0.25mm thickness and silica gel 60G. Capillary tube was filled by dipping one end into the extracts, and lightly applied to the thin layer plate. The ascending thin layer chromatography (TLC) was conducted in a wide chromatotank containing chloroform, ethyl acetate, methanol and water as the solvent system (15:8:4:1) The solvent system in the chamber was allowed to mix before loading the TLC plates.After that, the plates were allowed to dry under room temperature; 2cm width from the end of the TLC plates were cut out and sprayed with *para*-anisaldehyde for detection of the bands. The spots on the same line corresponding to the detected bands were scraped off from the unsprayed TLC plate and suspended in ethyl acetate solvent and centrifuged twice at 1500g for 10 min. The supernatants were concentrated to dryness at room temperature and reconstituted with DMSO. Each fraction obtained were tested for inhibitory activity against bacterial isolate as descried by (Nair *et al.,* 2005)., for zone of inhibition determination by agar well diffusion method and **minimum inhibitory concentration**MIC by 10 fold serial broth dilution method (Leboffe and Pierce, 1999). The active fraction was subjected to GC-MS analysis.

3.2.7. Phytochemical Screening of Fractions of TLC

The spray reagents method for Natural products TLC visualization was used as follows

3.2.7.1 Test for Terpenoids (Liebermann-Burchard Spray Reagent Test). Acetic anhydride (5ml) was carefully mixed under cooling with 5ml concentrated sulphuric acid; this mixture was added cautiously to 50ml absolute ethanol with cooling. After sparaying, the chromatogram was heated for 10minutes at 100^{o}C. (Takeda *et al.*,1963).

3.2.7.2 Test for Alkaloids (Dragendorff Spray Reagent Test)

Dissolve 10g tartaric acid in 50ml water and 10ml stock solution (equal volumes of solutions 1 and 2), APPENDIX 2.0.This forms the spraying solution. After spray, heat until dark orange-to-red coloration appears (Jork *et al.*, 1990)

3.2.7.3 Test forCardiac glycosides (Spray Test)

Procedure: Spray solution I (Sample A and B., APPENDIX 2.0, was sprayed lightly followed by spray solution II in excess. A blue violet colouration of the spot indicates the presence of cardiac glycosides(Tschesche*et al.*, 1953; Lewbart*et al*., 1963).

3.2.8Determination of Minimum Inhibitory Concentration (MIC)

The broth dilution method was used to determine MIC as described by (Leboffe and Pierce, 1999). A 3 times serial dilution was done as follows: 9ml of Muller Hinton broth was dispensed into each of the test tubes, then 1ml of 60µg/ml of ethyl acetate fraction was added and mixed properly. From this first test tube, 1ml of the mixture was taken and dispensed into the second test tube containing only 9 ml of the broth and mixed properly, then 1ml was as well taken from this second tube into the third tube also having 9ml of broth and was also mixed properly, then 1ml was finally taken out of this third tube and discarded. Finally 0.01ml of standardized test organism of bacterial cells which has been streaked over night on a Petri dish was added to each of the three tubes. Controls were equally set up using broth and test organisms only and the next control was the broth only.

Tubes were incubated aerobically for 24hours at 37°C. The plates were then observed for the presence or absence of growth. The concentration that completely inhibits macroscopic growth was regarded as the minimum inhibitory concentration of the extract.

3.2.9 Determination of Minimum Bacterial Concentration (MBC)

A sample from the tubes used in MIC (Minimum Inhibitory Concentration) determination which did notshow any visible growth after the period of incubation was streaked on Muller Hintonagar plates. The lowest concentration of the extract indicating a bactericidal effect after 24 hours of aerobic incubation at 37°C was regarded as the Minimum Bactericidal Concentration (MBC).

3.2.10. Fourier Transform Infra-Red spectroscopyAnalysis

The FTIR analysis of the TLC fraction with the highest MRSA inhibitory property was carried out to determine functional group(s) present using FTIR-8400S (Shimadzu model spectrophotometer), available at the National Research Institute for Chemical Technology (NARICT) Laboratory Zaria.

3.2.11. Identification of Active Component by Gas Chromatography/Mass Spectroscopy (GC-MS)

The active TLC bands of the ethyl acetate fraction of *Terminalia avicennioides*root-bark were dissolved in DMSO and were used for GC-MS.Aliquots of each sample were the injected into the sample inlet. The machine was programmed with syringe injection positionedat 250ºC temperature and 108.0 kPa pressures and at 80% column oven. As the separated substances emerge from the column opening, they flow into the Mass spectrometry which identifies compoundsby the mass of the analyte molecule. The machine is coupled to computer that has a library of known massspectra, covering several

thousand compounds which help to identify the likely compounds present in the sample. After that the print out of the result was obtained and used.

3.2.12. STATISTICAL ANALYSIS

The results are presented as mean ± standard deviation and differences between the means value zone of inhibition of the leaf and root sample were compared, and to the standard using ONE WAY ANOVA. A POST – HOC multiple test was used to compare the level of significance. P value > 0.05 was considered statistically non significant while P value < 0.05 was considered statistically significant.

CHAPTER FOUR

4.0 RESULTS

4.1. **Yieldof Successive Extraction of *Terminalia avicennioides*Leaf and Root Bark**

The yield of successive extraction of *Terminalia avicennioides*leaf and root bark extract is presented in Table 4.1.Methanol gave the highest yield at 12.66% followed by n-Hexane 1.18%, Ethyl acetate 1.75% and Chloroform 1.62%. While in root bark extraction, Methanol also gave the highest rate of yield at 13.01% value, followed by Chloroform 1.43%, which unlike in leaf extraction was the lowest in yield. N-Hexane gave 0.73% and Ethyl acetate 0.37%.

4.2 **MeanZone of Inhibition of Different Concentrations of the Leaf and Root Bark Extracts of *Terminalia avicennioides* on Methicillin Resistant *S. aureus***

The meanzone of inhibition of different concentrations of the leaf and root bark extracts of *Terminalia avicennioides* on methicillin resistant *S. aureus* is shown in Table 4.2 and graphically represented in Appendix 3.0. The values down the columnbearing the same alphabetical superscript are not significantly different($P>0.05$). Thus the Inhibitory activities observed across the extracts at all concentrations for both leaf and root bark are significantly lower ($P<0.05$) compared to the standard drug while there was no significant difference ($P>0.05$) in the activity of the leaf extracts at 60µg/ml and 120µg/ml. Similarly, at 180µg/ml and 240µg/ml activities were equally significantly lower ($P<0.05$) in all the extracts than it was in the standard. However, at 180µg/ml, activity in chloroform was significantly higher ($P<0.05$) compared to what was observed in ethyl acetate extract but not significantly different ($P>0.05$) from the inhibitory activities recorded in other extracts of the same concentration. Activities in chloroform extract at 240µg/ml was significantly higher ($P<0.05$) than the activity noticed in other extracts of the same concentration.

At 60µg/ml of the root bark extracts, the inhibitory activities did not differ significantly (P>0.05) from chloroform and n-Hexane extracts which both showed lower activities compared to what was observed in methanol and ethyl acetate extracts which also did not show a significant difference (P>0.05) in activities. At 120µg/ml, activity was significantly lower (P<0.05) in n-Hexane extract than what was observed in other extracts, where it appeared activity was higher in ethyl acetate extract compared to the activities in both methanol and chloroform extracts but only differ significantly (P<0.05) from the latter's. At 180µg/ml, the highest inhibitory activity was observed in methanol extract compared to others among which only chloroform extract showed a significant difference (P<0.05). At 240µg/ml, while there was no significant deference(P>0.05) observed between the inhibitory activity in methanol and chloroform extract, they both showed a significantly lower activity than what was observed in n-Hexane and ethyl acetate extracts in which the former showed a significantly lower activity.

Mean values in Roman numeral across the row at different concentrations describes a mean zone of inhibition of different concentration of the same extract for the leaf and for the root bark respectively. Roman numeral í is significantly higher than íí. (í > íí). The values in the leaf for each extract at different concentrations bearing the same superscript across the row are not significantly different(P>0.05). The same is for the rootbark. Thus Methanol, Ethyl acetate, and n-Hexane extract of the leaf had no significant difference (P>0.05) across all concentrations. Only Chloroform leaf extract at 60ug/ml shows a higher significant difference (P < 0.05). Similarly, all extracts of the root bark has no significant difference (P> 0.05).

Mean values in asterisks explains the comparison in the mean zone of Inhibition between theleaf and root bark at the same concentration and at the same solvent extract of *Terminalia avicennioides* on methicillin resistant *S. aureus.* Asteriks one is significantly higher ($P < 0.05$) than asterisk two than asterisk three (*>**>***).The values bearing the same superscript of asteriks are not significantly different ($P > 0.05$). Thus Methanol and Ethyl acetate extract of the root bark records significantly, a high zone of inhibition ($p < 0.05$) over its corresponding leaf extracts at the concentration of 60µg/ml and 120µg/ml respectively. The same is observed in ethyl acetate root bark and chloroform leaf extract at 180µg/ml. At 240µg/ml, Ethyl acetate and n-hexane root bark extracts are significantly higher ($p < 0.05$) than the corresponding leaf extracts while the leaf extract of chloroform is significantly higher ($p < 0.05$) than its corresponding root bark extract.

Table 4.1:**The Yield of Successive Extraction of *Terminalia avicennioides* Leafand Root Bark**

	Leaf		Root Bark	
Solvent	**Yield (g)**	**Yield (%)**	**Yield (g)**	**Yield (%)**
n-Hexane	3.54	1.18	2.18	0.73
Chloroform	1.62	0.54	4.29	1.43
Ethyl acetate	1.75	0.59	1.10	0.37
Methanol	37.98	12.66	39.01	13.01

Table 4.2 **Zone of Inhibition of Different Concentrations of the Leaf and Root Bark Extracts of *Terminalia avicennioides* on Methicillin Resistant *S. aureus***

4.3 Phytochemicals Present in the Most Bioactive Extract of *Terminalia avicennioides.*

Phytochemical present in Ethyl Acetate extract, the most bioactive extract of *Terminalia avicennioides* is presented in Table 4.3, it shows the presence of triterpens, alkaloids, tannin, cardiac glycoside, terpenoid, and anthraquinon, while flavonoids were absent.

4.4 Quantitative Estimation of Phytochemical Present in the Most Bioactive Extract of *Terminalia Avicennioides*

Quantitative estimation of phytochemicals present in Ethyl Acetate extract of the most bioactive extract of *Terminalia avicennioides* is presented in figure 4.1. It shows that 42µg of alkaloids,184.25µg of tannins and 19.80µg of anthraquinone were estimated in 1mg of root barkethyl acetate extract of *Terminalia avicennioides*. Calculations are presented in Appendix 4.0, 5.0 and 6.0

Table 4.3 **Phytochemicals Present in the Ethyl Acetate Extract of The Root Bark of*Terminalia avicennioides***

Phytohemical	
Triterpens	+
Alkaloids	+
Tannin	+
Flavonoid	–
Cardiac glycoside	+
Terpenoid	+
Anthraquinon	+

(+) = detected (-) = Not detected

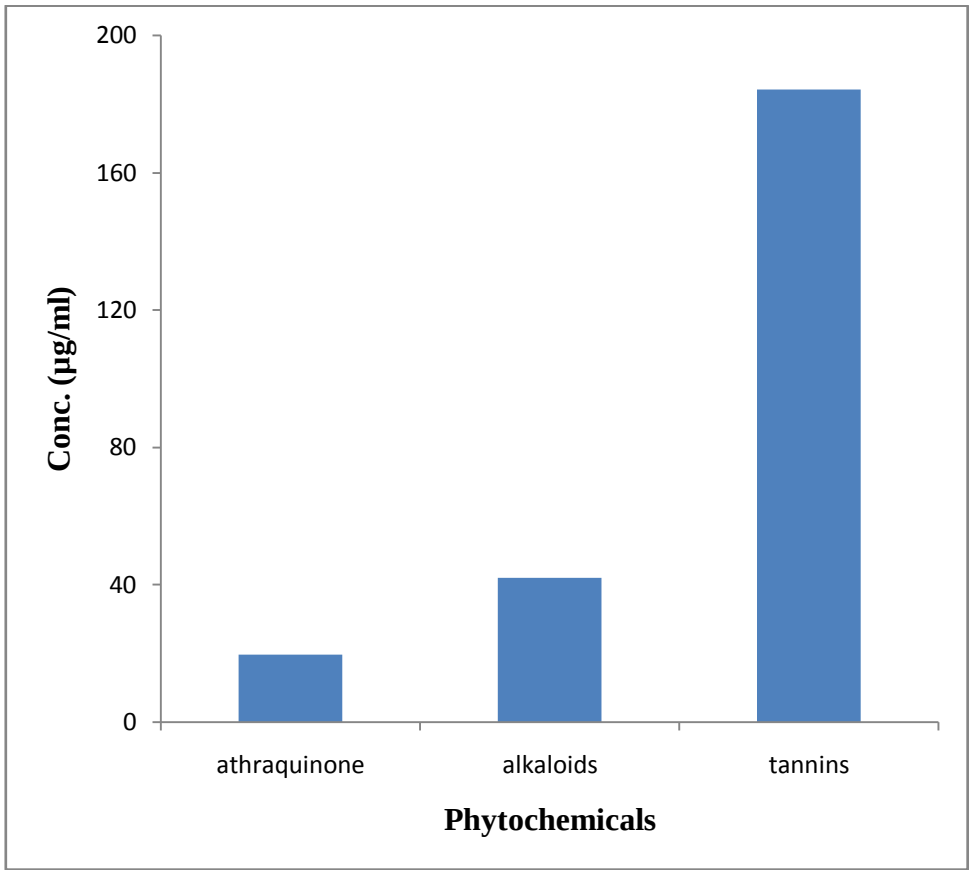


Figure 4.1**Quantitative Estimation of Phytochemicals Present in 1mg/ml Bioactive Ethyle Acetate Root Bark Extract of *Terminalia avicennioides***

4.5**Thin Layer Chromatogram of Root Bark Bioactive Ethyl Acetate Extract of*Terminaliaavicennioides.***

The TLC chromatogram of ethyl acetate extract of *Terminalia avicennioides*root barkis presented in plate 4.1. There were17 different visible bands with the 18th band too close to the solvent front using Chloroform: Ethyl acetate: Methanol: H_20 at (15:8:4:1) ratio.

4.6**Zone of Inhibition of Different TLCFractions of Root Bark BioactiveEthyl Acetate Extract of *Terminalia avicennioides***

The mean zone of inhibition in millimeter units of different TLC fractions of bioactive root barkethyl acetate extract of *Terminalia avicennioides* at concentration of 180µg/ml are shown inFigure 4.2. TLb4 and TLb17 being ≥ 14mm indicates sensitivity of Methicillin resistant *Staphylococcus aureus*withing the Kirby-Bauer standard for (Methicillin) antibiotic susceptibilityto the compounds in the bands.

4.7:**Mean Zone of Inhibition of Graded Concentrations of TLC Bands 4 and Band 17 of Root Bark Bioactive Ethyl Acetate Extract of *Terminalia avicennioides.***

The mean zone of inhibition in millimeter of graded concentrations of TLC bands 4 and band 17 of root barkbioactive ethyl acetate extract of *Terminalia avicennioides* is shown in Figure 4.3. The Sensitivity plates are shown in Appendix 6.0

4.8: **Phytochemicals Present in TLC Fractions ofRoot Bark BioactveEthyl Acetate Extractof*Terminalia avicennioide.***

The phytochemicals present in the TLC bands of the root bark of ethyl acetate extract of *Terminalia avicennioides* is presented in Table 4.4. Terpenoids were present in all bands except in band 2, 3, 5, 14 and band 16. Alkaloid was present in only band 6 and band 11, anthraquinone was present in band 10 only, while cardiac glycoside and tannins were present in band 18.

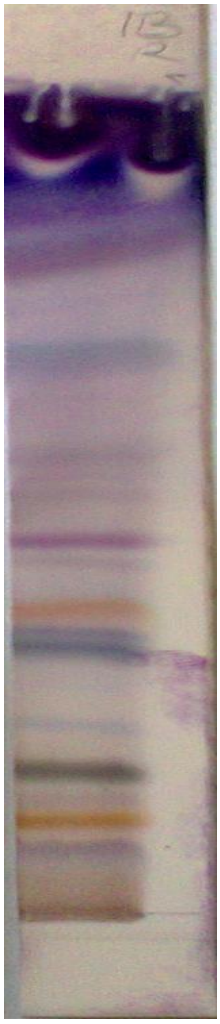


Plate 4.1 Thin Layer Chromatogram of ethyl acetate fraction of *Terminalia avicennioides* root barks developed with pre-coated TLC plate and a solvent system of Chloroform, Ethylacetate, Methanol and Water at ratio 15: 08: 04: 01

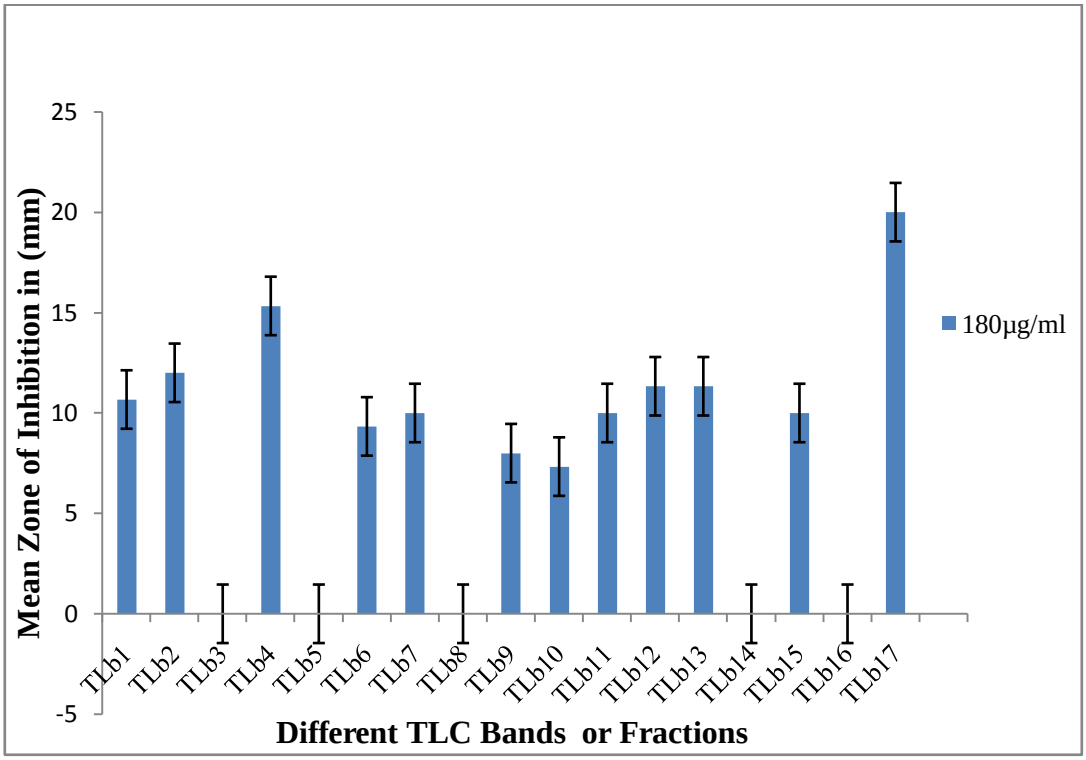


Figure4.2.Mean Zone of Inhibitionin (mm) of Different TLC Fractionsof Bioactive Root Bark Ethyl Acetate Extract of *Terminalia avicennioide* at 180µg/ml on Methicillin resistant *S. aureus*

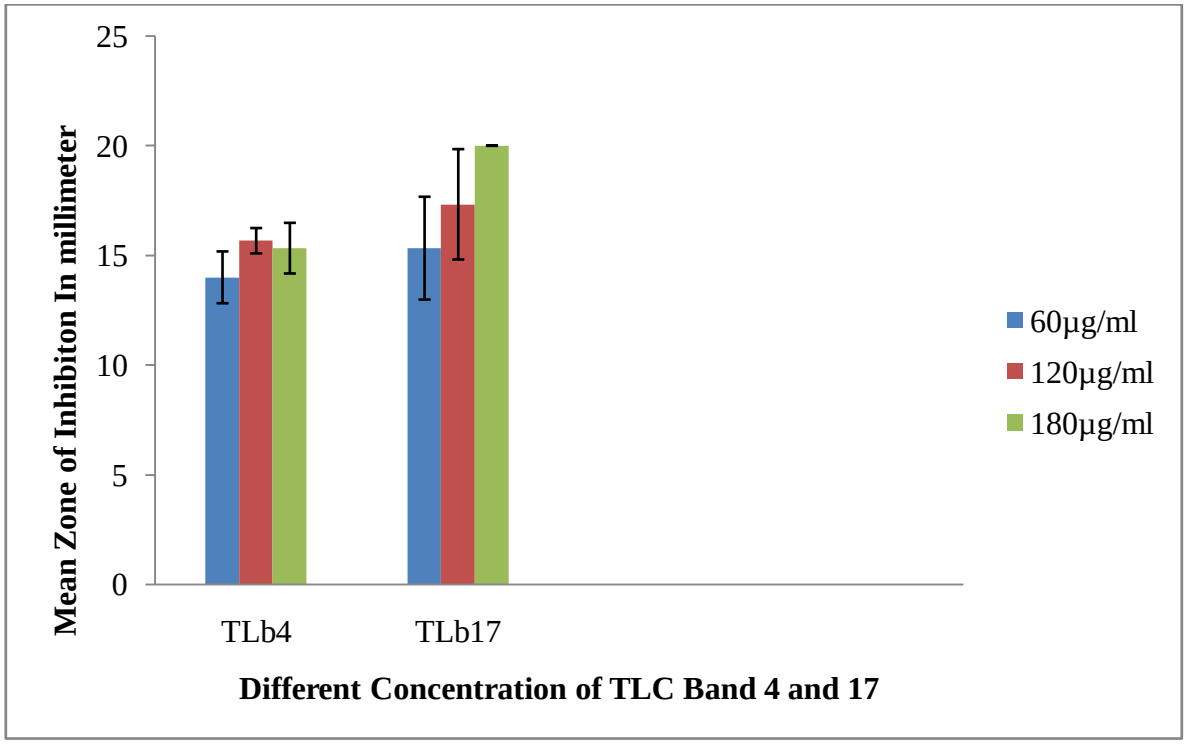


Figure 4.3 Mean Zone of Inhibition in (mm) of TLC Bands 4 and 17 of Ethyl Acetate Root Bark Extract of *Terminalia avicennioides* at Different Concentrations.

Table 4.4**Phytochemicals Present in TLC Bands of Root Bark of Ethyl Acetate Extractof *Terminalia avicennioides*using recommended Spray Reagents for Natural Product TLC Visualization Method.**

	Terpenoids	**Alkaloids**	**Anthraquinone**	**Cardiac glycosides**	**Tannins**
TLb1	+				
TLb2	ND				
TLb3	ND				
TLb4	+				
TLb5	ND				
TLb6	+	+			
TLb7	+				
TLb8	+				
TLb9	+				
TLb10	+		+		
TLb11	+	+			
TLb12	+				
TLb13	+				
TLb14	ND				
TLb15	+				
TLb16	ND				
TLb17	+				
TLb18	+			+	+

ND = Not Detected.

4.9 **Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of the Partially Pure Compound; TLC band 4 and TLC band 17 against MRSA**

The minimum concentration of the partially purified pure compound at which it is inhibitory to methicillin resistant *Staphylococcus aureus* was found to be 0.6µg/ml for both TLC band 4 and TLC band 17. It was as well bactericidal at the same concentration

4.10. **Gas Chromatography-Mass Spectroscopy Analysis of TLb4 and TLb17 Fraction of Root Bark BioactiveEthyl Acetate Extract of *Terminalia avicennioides***

Gas chromatography-mass spectroscopy analysis of bioactive TLb4 and TLb17 fractions of root bark ethyl acetate extract of *Terminaliaavicennioides* is presented in Table 4.5 and 4.6 respectively while the chromatograph is presented in Appendix 8.0.

Seven compoundswere present in TLb4 (Table 4.5) while six compounds were present in TLb17 (Table 4.6). These compounds are analog of palmitic acid (n-Hexadecanoic with molecular Weight (mol. weight) of 256,percentage (%) abundance of 3.95 and 1.52)., methyl ester (11-Octadecenoic acid with mol. weight of 296, % abundance of 2.90 and 1.82)., stearic acid (Octadecanoic acid with mol. weight of 284, % abundance of 4.07 and 2.97) and Oleic acid with mol. weight of 282, % abundance of 22.10 and 13.79, for both TLb4 and TLb17 fractions respectively.

4.11 FTIR **analysis for TLb4 and TLb17 Fraction of Ethyl Acetate Extract of Root Barks of *Terminalia avicennioides***

FTIR analysis for TLb4 and TLb17 fraction of ethyl acetate extract of root bark of *Terminalia avicennioides* is presented in table 4.7 and 4.8, while the spectroscopy pectrum is presented in Appendix 9.0. The functional groups detected includes Alkyl Halides: R-Br, R-F., Methyne: (CH−)., Nitro Compounds:R-NO2., Amides: R-C(O)-NH2, R-C(O)-NH-R., Isothiocyanate: (-NCS)., Nitril: C≡N., Carboxylic Acids., Saturated Aliphatic

(Alkyl):METHYLENE C-H., Alkanes., in both TLb4 and TLb17, while Alkyl Halide: R-I., Allenes and Amines: R-NH2 were found in addition to TLb17 only.

Table 4.5**Gas Chromatography Mass Spectroscopy Analysis of Fraction TLb4 of Bioactive Ethyl Acetate Root BarksEctract of *Terminalia avicennioides***

Table 4.6**Gas Chromatography Mass Spectroscopy Analysis of Fraction TLb17 of Bioactive Ethyl Acetate Root Barks Extract of *Terminalia avicennioides***

Table 4.7Major Peak Values in FTIR Analysis ofFractionTLb4of Boioactive Ethyl Acetate Root Bark Extract of*Terminaliaavicennioides*

S/No	Frequency range (cm^{-1})	Intensities	Assignment/ Bond	Functional Groups
1	399.28			
2	681.86	S	Aliphatic bromo compounds C-Br stretch	ALKYL HALIDES: R-Br
3	950.94		Cyclohexane ring vibrations	METHYNE (CH−)
4	1037.74	vs	C-F stretch	ALKYL HALIDES: R-F
5	1319.35	vs	C-F stretch	ALKYL HALIDES:R-F
			=C-O-C sym. & asym. stretch	ETHERS Ar-O-R
6	1422.55	S	N-O symmetric & asym. stretch	NITRO COMPOUNDS: R-NO2
7	1654.98	s broad	Carbonyl C=O stretch	AMIDES: R-C(O)-NH2 R-C(O)-NH-R
8	1990.60	m-s	Isothiocyanates (-NCS)	ISOTHIOCYANATE
9	2099.59	m-s	Isothiocyanates (-NCS)	ISOTHIOCYANATE
10	2330.09	m	C≡N	Nitril
11	2602.06	s broad	O-H stretch	CARBOXYLIC ACIDS
12	2918.40	V	*sp3* C–H strech	SATURATED ALIPHATIC (ALKYL):METHYLENE C-H
13	2997.48	S	*sp3*c-H stretch	ALKANES
14	3431.48	w-m	N-H asym. Stretch	AMIDES: R-C(O)-NH2
15	3922.38	88.849		
16	4022.68	88.829		
17	4291.76	86.117		
18	4402.67	84.075		

Intensity abbreviations: vw = very weak, w = weak, m = medium, s = strong, vs = very strong.

Table 4.8 MajorPeak Values in FTIR Analysis ofFractionTLb17of Bioactive Ethyl Acetate Root Bark Extract of *Terminalia avicennioides*

S/No	Frequency range (cm^{-1})	Intensities	Assignment/ Bond	Functional Groups
1	441.71	s	C-I stretch	ALKYL HALIDES: R-I
2	681.86	s	Aliphatic bromo compound C-Br stretch	ALKYL HALIDES: R-Br
3	951.90		Cyclohexane ring vibrations	METHYNE (CH−)
4	1033.92	vs	C-F stretch	ALKYL HALIDES: R-F
5	1320.32	vs	C-F stretch	ALKYL HALIDES: R-F
				ETHERS
		m-s	=C-O-C sym. & asym. stretch	Ar-O-R
6	1423.51	S	N-O symmetric & asym. stretch	NITRO COMPOUNDS: R-NO2
7	1655.94	s broad	C=O stretch	AMIDES: R-C(O)-NH2 R-C(O)-NH-R
8	1987.71	m-s	Allenes, Ketenes, Isocyanates, Isothiocyanates	X=C=Y
9	2140.09	m-s	Isothiocyanates (-NCS)	ISOTHIOCYANATE
10	2337.80	m	C≡N	Nitril
11	2921.29	S	C-H stretch	ALKANES
12	3003.27	s, broad	O-H stretch	CARBOXYLIC ACIDS
			H-bonded	Alcohols, phenols
13	3422.80	w	N-H asym. stretch	AMINES: R-NH2
	3935.88	86.26		
	4025.57	86.307		
	4295.62	84.862		
	4404.6	83.452		

Intensity abbreviations: vw = very weak, w = weak, m = medium, s = strong, vs = very strong.

CHAPTER FIVE

5.0 DISCUSSION

It is no news that plants produce a plethora of bioactive secondary metabolites drawing many curious researchers to the forest of herbs. This search for inhibitory potential of leaf and root bark extracts and characterization of thedistinct bioactive extract from one of the most unexplored forest plants; *Terminalia avicennioides* has reveled that chloroform is the best solvent in inhibitory activity wise for extraction of bioactive components in leaf of the plant material compared to other solvents as shown in (Table 4.2 and Appendix 3.0)., as it maintained a concentration dependent increasing inhibitory activity anddespite having the lowest of mean zone inhibition at 60µg/ml, it maintained the widest mean zone of inhibition across the graded concentrations of 120µg/ml, 180µg/ml and 240µg/ml.

Nevertheless, ethyl acetate was determined to be the best solvent for extraction of bioactive principles in the root bark of *Terminalia avicennioides* considering its high inhibitory activity when compared across the inhibitory activities of all concentrations of other solvents used in the extraction (Table 4.2) and as well when compared to the Chloroform activity of the leaf as shown in (Table 4.2). This led to further studies on ethyl acetate root bark extract. However, this finding is in contrast with (Mann *et al*., 2009) use of root portion, whose final findings is that fractions of n-hexane gave higher activity over fractions of ethyl acetate extract, though both n-hexane and ethyl acetate extractgave significantly higher activity ($P < 0.05$) over acetone and methanol extracts. This contrast may be due to the fact that root bark portion was used in this study. The result indicates that the ethyl acetate root bark extraction has definite effect among other solvents for the isolation of bioactive principles in the root bark of *Terminalia avicennioides* and could

probably be due to it polarity index of 4.4 (intermitently polar) thus having the ability of extracting series of saturated and unsaturated fatty acids with antimicrobial abilities like oleic acid etc., as found in the GCMS analysis and as well some other possible antimicrobial components not detectable by GCMS analysis .

According to Prescott *et al.,* 2002, the activity of antimicrobial agent is concentration dependent. (Hugo and Russell, 1998) also indicated that the position of the zone edge (diameter of inhibition) is determined by the initial population density of the organism, their growth rate and the rate of diffusion of the antimicrobial agent. These two principles were obeyed by all the leaf extracts (Table 4.2) whose mean zone of inhibitions recorded an increase as concentration increased, but however arriving at a concentration of 180µg/ml with the exception of ethyl acetate extract at 120µg/ml, further increase in concentration gave no further increase in activity. This observation could be due to the presence of high molecular weight fatty acids and probably other components whose rate of diffusion could be limited by entrapment or adhesion of such compound on the agar gel bits and probably would not have been entrapped or adhered to the agar gel bits were they to be of low molecular weights, volatile oil or even of gaseous components. The observation could as well be due to initial population density of the organism under study as indicated by (Hugo and Russell, 1998). Similarly, the same principle was followed but not completely by root barks extract as only methanol extract increased in activity as concentration increases and got to maximum at 180µg/ml where further increase in concentration caused no further increase in activity. Chloroform extract of root bark only had a maximum increase in activity at 120µg/ml and increased no further as concentration increases. Irrespective of some of these irregularities in parttern of the results it is most

interesting that with the exception of only chloroform, ethyl acetate and n-Hexane extract of the leaf at 60µg/ml, every other extracts of the leaf and root bark at all concentrations are inhibitory to the test organism within the susceptible range (≥ 14mm) as defined by Kirby-Bauer standard chart for (Methicillin) antibiotic susceptibility.

The inhibitory activity believed to be exhibited by these secondary metabolites (phytochemicals present., Table 4.3) tends to agree with the reports of Scherbanvoski (1971) who linked the antibacterial properties of plants to the presence of secondary metabolites and (Mann *et al*., 2008) in whose conclusion the presence of the secondary metabolites in *Terminaliaavicennioides* may be responsible for its potential use asdrugs.

Sensitivity test of all TLC bands of the root bark ethyl acetate extract of *Terminaliaavicennioides*shows that only TLb4 and TLb17 had inhibitory activity on MRSA within the susceptible range (≥ 14mm).Terpenoids where the most phytochemical metabolite present, in 13 out of the 18 fractionsin TLb1 to TLb18 fractions of ethyl acetate root bark extract (Table 4.6). Though TLb18 possesses the presence of terpenoids, cardiac glycosides and tannins, it was not tested for activity as the band was too close to the solvent front. TLb6 and TLb11 recorded the presence of both terpenoids and alkaloids while TLb10 showed the presence of terpenoid and anthraquinone but on the whole, showed no significant inhibitory activity on MRSA.

In quantitative estimation of phytochemicals,42µg of alkaloids, 19.80µg of anthraquinone and 184.25µg of tannins were estimated in 1mg/ml, of ethyl acetate root bark extract of *Terminalia avicennioides.* This is the first time quantitative phytochemical estimation is done on any part of this plant.

MIC value was observed to be 0.6µg/ml for both TLb4 and TLb17 and as well bactericidal at this same concentration.

Functional group maintains the core of anti microbial activity compounds. The presenceof alkyl halides: R-Br, R-F., Methyne: (CH−)., Nitro Compounds:R-NO2., Amides: R-C(O)-NH2, R-C(O)-NH-R., Isothiocyanate: (-NCS)., Nitril: C≡N., Carboxylic acids., Saturated aliphatic (Alkyl):METHYLENE C-H., Alkanes., in both TLb4 and TLb17, and Alkyl halide: R-I., Amines: R-NH2 in TLb17 which comfirms the pharmacological activities observed in these fractionsas further justified by work done by other authors.Findings by Hollosy *et al*., (2002) and Litvin *et al*., (2004) state that heteroatom, in particular halogen, substitution on aryl ring substituents frequently produced compounds with improved biological activity and the incorporation of halogen atoms into drug candidates have generally been correlated with increased lipophilicity, which may allow improved penetration through cell lipid membranes. This may explain the process of inhibiton by the presence of the alkyl halids in the fractions. Compounds based around nitrogen-containing heterocyclic frameworks (Wyatt *et al*., 2008), amides in form of diamides(Pauk *et al*., 2013 and Zadrazilova *et al*., 2014), dominates as the active species compounds that exhibited a great anti-MRSA with dihydrofolate reductase inhibitory activity. isothiocyanates from cruciferous plants has shown a very strong anti-MRSA activity of up to 87% with MIC value between 2.9 and 110 µg/mland were mainly bactericidal (Dias *et al*., 2014) Ether functional groups available in biphenyl ether, antibacterial is implicated in exhibition of broad–spectrum antibiotics activity targeting the fatty acid biosynthetic pathway, *via* inhibition of enoyl–acyl carrier protein reductase (ENR). (Tipparaju *et al*., 2008). Likewise in vancomycin (Rybak *et al*., 2009).

The result obtained from GC-MS analysis showed that the likely active compounds were analogs of palmitic acid (n-Hexadecanoic), methyl ester (11-Octadecenoic acid), stearic acid (Octadecanoic acid) and Oleic acid for both TLb4 and TLb17 fractions respectively. However, it is possible that other compounds or group of compounds present in the extract, although not suspected to have elicited the inhibitory effects may have inhibited the growth of MRSA or contributed to the observed inhibition. This is because of the presence of 9-Octadecenal and 3, 11-Tetradecadien-1-ol in both TLb4 and TLb17 fractions respectively, and (9Z)-9-Teradecenal present only in TLb4 fraction of the root barks bioactive ethyl acetate extract of *Terminalia avicennioides.*

The antimicrobial activity and therapeutic efficacy of oleic acid against methicillin-resistant *S. aureus* have been proven (Huang *et al*., 2011). Fatty acids, particularly methyl esterpossess antibacterial activities (Chandrasekaran *et al*., 2008). Palmitic acid, stearic acid and many other fatty acids have been reported to have some antimicrobial activity against MRSA (Gheda1 *et al*., 2013). (9Z)-9-Teradecenal has also been identified among fatty acids with antimicrobial activities against MRSA (Ololade *et al*., 2014). Meanwhile, this is the first time 3, 11-Tetradecadien-1-ol is found among compounds with anti MRSA potential.

Potential antimicrobial activity is attributed to different compounds belonging to a diverse range of chemical classes (Ozdemir *et al*., 2004). Oleic acid has the highest % abundance with value of 22.10 and 13.79, for both TLb4 and TLb17 fractions respectively, and study has demonstrated that oleic acid effectively eliminates *Staphylococcus aureus* through cell wall disruption and can inhibit growth of a number of Gram-positive bacteria, including hospital and community-associated MRSA (Chen *et al*., 2011), therefore, we speculate that

the anti MRSA effect of TLb4 and TLb17 fractions of the root bark bioactive ethyl acetate extract of *Terminalia avicennioides* might highly be due to the presence of oleic acid.

As a known hypothesis, fatty acids interferes with the bacterial cell structure and acids interfere with cellular energy production, causing disruption of the electron transport chain and oxidative phosphorylation. (Carpo *et al*., 2007 and Parfene *et al*., 2013). Fatty acids may also inhibit enzyme activity, impair nutrient uptake, generate cellular degradation products, or cause direct lysis of infectious cells. (Parfene *et al*., 2013 and Carpo *et al*., 2007), and some fatty acid compounds with a small percentage have been reported to have significant antimicrobial activity (Hashem and Saleh, 1999). These components at low concentrations might be involved in some type of synergism with the other active compounds (Marino *et al*., 2001) thus exhibiting high antibacterial activities on MRSA. Therefore it would be safe to conclude that palmitic acid (n-Hexadecanoic), methyl ester (11-Octadecenoic acid), stearic acid (Octadecanoic acid), with 9-Octadecenal, 3, 11-Tetradecadien-1-ol and (9Z)-9-Teradecenal, all which have a low % abundance could be inhibiting based on the above mechanism.

Nevertheless, it is worthy to note that the antimicrobial activities of plant extract are dependent on the rate of diffusion of the extract. These low-molecular weight and highly lipophilic compounds easily diffuse across cell membranes to induce biological reactions. Also, if the extract has high molecular weight, the rate of diffusion is always slow, reduced and also takes longer time, whereas an extract of low molecular weight diffuses faster and at a quicker rate (Orji *et al*., 2012). However, it is possible that the compounds with high molecular weight although diffuses at a slow rate may elicit a stronger biological effect.

Secondary metabolites of non fatty acid nature in plant extract inhibits the life processes of microorganisms by binding their protein molecules, acting as chelating agents, altering their biochemical systems, or causing inflammation of the cells. Example, terpenes promote membrane disruption, and tannins act on microorganism membranes as well as bind to polysaccharides or enzymes promoting inactivation (Cowan, 1999). In this regard the organic compounds in this medicinal plant would play an important role as a good natural source of antibiotics for further studies.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 SUMMARY

- Leaf and root bark of *Terminalia avicennioides* were extracted with four different solvents giving a leaf extracts yield of 37.98g (12.66%,)., 3.54g (1.18%)., 1.75g (1.75%)., 1.62g (1.62%) for Methanol, n-Hexane, Ethyl acetate and Chloroform respectively while root bark extracts yielded 39.01g (13.01%)., 4.29g (1.43%)., 2.18g (0.73%)., 1.10g (0.37%) for Methanol, Chloroform, n-Hexane and Ethyl acetate respectively.
- Antimicrobial screening of the leaf and root bark extracts of *Terminalia avicennioides* on methicilin resistant *staphylococcus aureus* was done using the four different extracts for the leaf and root bark at graded concentrations of 60µg/ml,120µg/ml,180µg/ml and 240µg/ml. Chloroform extract of the leaf and Ethyl acetate extract of the root bark were observed to be the best system for isolation of bioactive principles and maintained a significant inhibitory records within the susceptible range (≥ 14mm) as defined by Kirby-Bauer standard chart for (Methicillin) antibiotic susceptibility on the test organism with only the exception of only Chloroform, Ethyl acetate and n-Hexane extract of the leaf at 60µg/ml
- Antimicrobial studies of inhibitory activities of Chloroform leaf extract and Ethyl acetate root bark extract led to further investigation of Ethyl acetate extract of the root bark.

- Amongst the tested phytochemical has the presence of Triterpenes, Alkaloids, Tannin, Cardiac glycoside, Terpenoid, and Anthraquinon, while Flavonoid was found to be absent in root bark of ethyl acetate extract.
- Further investigation of Ethyl acetate extract of the root bark gave fraction TLb1-18 in which fractions TLb4 and TLb17gave significant activity thus were characterized for bioactive principles and functional group present, using GC-MS and FTIR spectroscopy respectively.
- Phytochemical analysis of the fractions of root bark ethyl acetate extract using recommended spray reagents for natural product TLC visualization method indicated that Terpenoids were present in all Fractions except in TLb 2, 3, 5, 14 and 16. Alkaloids were found present in only TLb6 and TLb11, Anthraquinone was present in TLb10 only, while Cardiac glycoside and Tannins were present in TLb18.
- FTIR analysis of fractions TLb4 and TLb17showed that these functional groupsAlkyl Halides: R-Br, R-F., Methyne: (CH−)., Nitro Compounds:R-NO2., Amides: R-C(O)-NH2, R-C(O)-NH-R., Isothiocyanate: (-NCS)., Nitril: C≡N., Carboxylic acids., Saturated aliphatic (Alkyl):METHYLENE C-H., Alkanes., were present in both TLb4 and TLb17, while Alkyl halide: R-I., Allenes and Amines: R-NH2 were found present in addition to TLb17 only.
- GC-MS analysis of fractions TLb4 and TLB17 showed that the most likely compound responsible for the inhibitory effects were majorly Oleic acidwith mol. Weight of 282, abundance of 22.10% and 13.79%, for both TLb4 and TLb17 fractions respectively., analogs of palmitic acid (n-Hexadecanoicwith mol. Weight

of 256, abundance of 3.95% and 1.52%)., methyl ester (11-Octadecenoic acidwith mol. Weight of 296, abundance of 2.90% and 1.82%)., stearic acid (Octadecanoic acidwith mol. Weight of 284, abundance of 4.07% and 2.97%) in a possible synergisim with (9-Octadecenalwith mol. Weight of 266,abundance of 4.39% and 3.73%)., (3,11-Tetradecadien-1-ol mol. Weight of 210, abundance of 3.08% and 0.81%) in both TLb4 and TLb17 fractions respectively, and (9Z)-9-Teradecenalwith mol. Weight of 210 and abundance of 1.47% present only in fraction TLb17.

- This study establishes that: ethyl acetate extract of root bark of *Terminalia avicennioides*at a dose of 120µg/ml in vitro, had significant ($P < 0.05$) inhibitory effect on the growth of methicillin resistant Staphylococcus aureus. Level of inhibition was 72.4% of that by augumentin; the standard drug. The ethyl acetate extract of the leaf of this plant was also inhibitory to the growth of methicillin resistant Staphylococcus aureus but less effective than the root bark extract (level of inhibition was 56.24% of that to augumentin.

6.2 CONCLUSION

This study has scientifically validated the ethno medical use of leaf and root bark extracts of*Terminalia avicennioides* by the locals in the treatment of skin infections particularly caused by the test organism and has as well revealed that the root bark has higher inhibitory potential over the leaf. Findings from the GCMS characterization of the root bark fractions suggests that the identified bioactive compounds fatty acids majorly oleic acid alone and a possiblesynergisim with analogs of palmitic acid, methyl ester, stearic acid with 9-

Octadecenal,3, 11-Tetradecadien-1-ol and(9Z)-9-Teradecenal are effective therapeutic agents for the treatment and management of disease conditions caused by methicillin resistant *Staphylococcus aureus*

6.3 RECOMMENDATIONS

i. Considering these findings, it is therefore recommend that these natural source fatty acids are harnessedin industrial amounts towards maximum development of standard drug and dosage for the treatment of medical conditions of methicillin resistant Staphylococcus aureus significance.

ii. Due to the limitations of GC-MS, as it detects only volatile fatty acid at its operational high temperature, there is need for further studies on these bioactive ethyl acetate fractions of the root bark extract of the plant using LCMS and HPLC for a possible detection of some other compounds capable of being active on the test organism

iii. Futher studies should be carried out to check the effect of these extracts on *mecA* gene that confers resistance to MRSA

iv. Further research should be carried out on the In-vivo studies to ascertain the effects of the bioactive compounds of the extract on the organism.

REFERENCE

Abdalla, E.M. (2011). Plants: An alternative source for antimicrobials. *Journal of Applied Pharmaceutical Science*, 1(6): 16-20.

Abdullahi, A.L., Agho, M.O., Amos, S., Gamaniel, K.S. and Wambebe, C. (2001). Antidiarrhoeal activity of the aqueous extract of *Terminalia avicennioides* roots. *Phytother. Res*, 15, 431-434.

Aires de Sousa, M., and de Lencastre, H.(2003). Evolution of sporadic isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) in hospitals and their similarities to isolates of community-acquired MRSA. *Journal of Clinical Microbioogyl*; 41:3806–15.

Ajoke, O. I., Ikenna, O.O., Olumide, A. O., and Okwori A.E.J. (2012). Prevalence of methicillin-resistant staphylococcus aureus from healthy community individuals volunteers in jos south,Nigeria. *Journal of Microbiology,Biotechnology*1 (6) 1389-1405.

Akinside, K.A. and Olukoya, D.K. (1995). Vibrocidal activities of some local herbs. *Journal of Diarhoeal Disease Research,*13:127-129.

Akinyemi, K. O., Olukayode, O., Okwara, C. E., Ibe, C. C., and Fasure K. A., (2005). Screening of crude extracts of six medicinal plants used in South-West Nigerian unorthodox medicine for anti-methicillin resistant *Staphylococcus aureus* activity.*BMC Complementary and Alternative Medicine,* 5:6 doi: 10.1186/1472-6882-5-6.

Akinyemi, K.O., Bayagbon, C., Oyefolu, A.O.B., Akinside, K.A., Omonigbeyin, E.A. and Coker, A.O. (2000). Antibacterial screening of five indigenousNigerian medicinal plants against *S. typhi* and *S. paratyphi*.*Journal of Nigerian infection control association*, 3(1):30-33.

Akinyemi, K.O., Mendie, U.E., Smith, S.T., Oyefolu, A.O., and Coker, A.O. (2005). Screening of some medicinal plants used in south-west Nigerian traditional medicine for anti-Salmonella typhi activity. *J Herb Phamacother,* 5:45-60.

Alam, M.T., Karim, M.M. and Khan, S.N. (2009). Antibacterial activity of different organic extracts of *Achyranthes aspera* and *Cassia alata*. *Journal of Scientific Research*., 1(2): 393-398.

Aliyu, A. B., Musa, A. M., Abdullahi, M. S., Oyewale, A. O. and Gwarzo, U. S. (2008). Activity of plant extracts used in Northern Nigerian traditional medicine against methicillin-resistant *staphylococcus aureus* (MRSA). *Nigerian Journal of Pharmaceutical Sciences* Vol. 7, 1: 1– 8.

Archer, G.L., and Bosilevac, J.M. (2001). Signaling antibiotic resistance in staphylococci. *Science*, 291:1915–6.

Atawodi, S.E., Ameh, D.A., Ibrahim, S., Andrew, J.N., Nzelibe, H.C., Onyike, E.O., Anigo, K.M., Abu, E.A.,James, D.B., Njoku, G.C., & Sallau, A.B. (2002). Indigenous knowledge system for treatment of trypanosomiasisin Kaduna state of Nigeria. *Journal of Ethnopharmacolology*, 79, 279-28.

Atawodi, S. E., Bulus, T., Ibrahim, S., Ameh, D.A., Nok, A.J., & Mamman, M. Galadima, M. (2003). *In vitro* trypanocidal effect of methanolic extract of some Nigerian Savannah plants. *African Journal Biotechnology*, 2(9),317-321.

Autiero, I., Costantini, S., Colonna, G. (2009). Modeling of the Bacterial Mechanism of Rethicillin-Resistance by a Systems Biology Approach. PLoS ONE 4(7): e6226. doi:10.1371/journal.pone.0006226.

Baba, T., Takeuchi, F., Kuroda, M., Yuzawa, H., Aoki, K., Oguchi, A., Nagai, Y., Iwama, N., Asano, K., Naimi, T., Kuroda, H., Cui, L., Yamamoto, K., Hiramatsu, K. (2002). Genome and virulence determinants of high virulence community-acquired MRSA. *Lancet*, 359:1819–27.

Boyce, J.M., Potter-Bynoe, G., Chenevert, C., and King, T. (1997). Environmental contamination due to methicillin-resistant Staphylococcus aureus: possible infection control implications. *Infect Control Hosp Epidemiol*, 18:622-627.

Burdette, S. D., Watkins, R. R., Wong, K. K., Mathew, S. D., Martin, D. J. and Markert, R. J. (2012). Staphylococcus aureus pyomyositis compared with non-Staphylococcus aureus pyomyositis. *J Infect*, 64, 507–512.

Burkill, H.M. (1985). The useful plants of Tropical Africa, 2nd Ed., Royal Botanic Gardens, Kew, London, pp. 389-391; 416-417.

Carpo, B.G., Verallo-Rowell, V.M., Kabara, J. (2007). Novel antibacterial activity of monolaurin compared with conventional antibiotics against organisms from skin infections: an in vitro study. *J Drugs Dermatol*. 6(10):991-998.

Centre for Disease Control and Prevention (2003). National Nosocomial Infections Surveillance (NNIS) System report. (2003).*Am. J. Infect. Contr.*, 31, 481-498

Centre for Disease Control and Prevention (2004). National Nosocomial Infections Surveillance (NNIS) System report. (2004). Data summary from January 1992 through June 2004. *Am. J. Infect. Contr.*, 32, 470-485.

Chambers, H.F., Sachdeva, M.J., and Hackbarth, C.J. (1994). Kinetics of penicillin binding to penicillin-binding proteins of Staphylococcus aureus. *Biochem J* 301: 139–44.

Chambers, H. F. (2001). The changing epidemiology of Staphylococcus aureus? *Emerg Infect Dis*, 7:178-182.

Chambers, H.F., and Deleo, F.R. (2009). Waves of resistance: Staphylococcus aureus in the antibiotic era. *Nat Rev Microbiol* 7: 629–641.

Chanda, S., Vyas, B.R.M., Vaghasiya, Y., and Patel H. (2010). Global resistance trends and the potential impact of Methicillin Resistant *Staphylococcus aureus* (MRSA) and its solutions; Current Research, Technology and Education in Applied Microbiology and Microbial Biotechnology A. Mendez-Vilas (Ed.)

Chandrasekaran, M., Kannathasan, K., and Venkatesalu, V. (2008). "Antimicrobial activity of fatty acid methyl esters of some members of chenopodiaceae," *Zeitschrift fur Naturforschung C*, vol. 63, no. 5-6, pp. 331–336.

Changchien, C. H., Chen, Y. Y., Chen, S. W., Chen, W. L., Tsay, J. G. and Chu, C. (2011). Retrospective study of necrotizing fasciitis and characterization of its associated methicillin-resistant Staphylococcus aureus in Taiwan. *BMC Infect Dis*, 11, 297.

Chen, C.H., Wang, Y., Nakatsuji, T., Liu, Y.T., Zouboulis, C., Gallo, R., Zhang, L., Hsieh, M.F., Huang, C.M.(2011). An innate bactericidal oleic acid effective against skin infection of methicillin-resistant Staphylococcus aureus: a therapy concordant with evolutionary medicine. *J Microbiol Biotechnol*. 21(4):391-9.

Clinicaland Laboratory Standards Institute (2007). Performance Standards for Antimicrobial Susceptibility Testing; Seventeenth Information Supplement. M100-S17; Vol. 27 No. 1

Coello, R., Jiménez, J., García, M., Arroyo, P., Minguezl, D., Fernández, C., Cruzet, F., Gaspar, C.(1994). Prospective study of infection, colonization and carriage of methicillin-resistant Staphylococcus aureus in an outbreak affecting 990 patients. Eur J Clin Microbiol Infect Dis, 13:74-81.

Cowan, M.M. (1999). Plant products as antimicrobial agents. *Clinical Microbiology Reviews*. 12:564-582.

Dias, C., Aires, A., Saavedra, M. J. (2014). Antimicrobial Activity of Isothiocyanates from Cruciferous Plants against Methicillin-Resistant *Staphylococcus aureus* (MRSA)*Int. J. Mol. Sci*, 15: 19552-19561.

Donnio, P.Y., Preney, L., Gautier-Lerestif, A.L., Avril,J-L.,Nathalie, L. (2004). Changes in staphylococcal cassette chromosome type and antibiotic resistance profile in methicillin-resistant *Staphylococcus aureus* isolates from a French hospital over an 11 year period. *Journal of Antimicrobial Chemotherapy*, 53: 808–13.

Drews, T.D., Temte, J.L., and Fox, B.C. (2006). Community-associated methicillin-resistant *Staphylococcus aureus*: Review of an emerging public health concern. *Wisconsin Medical Journal*, 105:52-57.

Eloff, J.N. (1998a). Conservation of Medicinal Plants: Selecting Medicinal Plants for research and gene banking. Monographs in systemic Botany from the Missouri

Garden 71, 209-222 *In*: Conservation of plants Genes III: Conservation and utilization of African plants. Robert, P., Adams and Janice E. Adams, eds., Missouri Botanical Garden Press, St. Louis, USA.

Eloff, J.N. (1998b).Which extractant should be used for screening and isolation of antimicrobial components from plants? *Journal of Ethnopharmacology*, 60, 1-8.

Ender, M., McCallum, N., Adhikari, R., and Berger-Bãchi, B. (2004). Fitness cost of SCC*mec* and methicillin resistance levels in *Staphylococcus aureus.Antimicrob Agents Chemother*, 48:2295–7.

Enright, M.C., Day, N.P., Davies, C.E., Peacock, S. J., Spratt, B. G. (2000). Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptibile clones of *Staphylococcus aureus. Journal of Clinical Microbiology*, 38: 1008–15.

Enright, M.C., Robinson, D.A., Randle, G., Feil, E.J., Grundmann, H., Spratt, B.G. (2002). The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). *Proceedings of the National Academy of Sciences of the United States of America*, 99:7687-7692.

Evan, W.C. (1989). Trease and Evans Pharmacognosy. 13th Edition, Bailere Traiadal, London. pp. 101-104.

Eveillard, M., Martin, Y., Hidri, N., Boussougant, Y., and Joly-Guillou, M. L. (2004). Carriage of methicillin resistant Staphylococcus aureus among hospital employees: prevalence, duration, and transmission to households. *Infect Control Hosp Epidemiol*, 25:114-120.

Francis, J. S., Doherty, M. C., Lopatin, U., Johnston, C. P., Sinha, G., Ross, T., Cai, M., Hansel, N. N., Perl, T. and other authors (2005). Severe community-onset pneumonia in healthy adults caused by methicillin-resistant Staphylococcus aureus carrying the Panton- Valentine leukocidin genes. *Clin Infect Dis*, 40, 100–107.

Fuda, C. C. S., Fisher, J. F. and Mobashery, S. (2005). Beta-lactam resistance in *Staphylococcus aureus*: the adaptive resistance of a plastic genome. *Cell. Mol. Life Sci*. 62:2617–2633.

Fuda, C., Suvorov, M., Vakulenko, S.B., Mobashery, S. (2004). The basis for resistance to b-lacatm antibiotics by penicillin-binding protein 2a (PBP2a) of methicillin-resistant *Staphylococcus aureus. J Biol Chem* 279:40802–6.

García Álvarez, L., Webb, C. R., and Holmes, M. A. (2011a). A novel field-based approach to validate the use of network models for disease spread between dairy herds. *Epidemiol. Infect*. 139, 1863–1874.

García-Álvarez, L., Holden, M. T.G., Lindsay, H., Webb, C. R., Brown, D. F. J., Curran, M. D., Walpole, E., Brooks, K., Pickard, D. J., Teale, C., Parkhill, J., Bentley, S. D., Edwards, G. F., Girvan, E. K., Kearns, A. M., Pichon, B., Hill, R. L. R, Larsen, A. R., Skov, R. L., Peacock, S. J., Maskell, D. J., and Holmes, M. A.

(2011b). Meticillin-resistant *Staphylococcus aureus* with a novel mecA homologue in human and bovine populations in the UK and Denmark: a descriptive study. *Lancet Infect. Dis.* 11, 595–603.

Gheda1, S. F., Khalil, M. A., and Gheida, S. F. (2013). In vitro and in vivo preliminary results on Spirulina platensis for treatment of impetigo: Topical cream application. Vol. 12(18), pp. 2498-2509

Ghosh, A. S., Chowdhury, C., and Nelson, D. E. (2008). Physiological functions of D-alanine carboxypeptidases in *Escherichia coli*. *Trends Microbiol.*16:309–317.

Gill, L.S. and Akinwunmi, C.(1986). Nigerian Medicine Practice and Beliefs of *the* Ondo People. *J Ethnopharmacol*, 18:257-266.

Gordon, R.J., Lowy, F.D. (2008). Pathogenesis of methicillin-resistant Staphylococcus aureus infection. *Clin Infect Dis*, 46 Suppl 5: S350–9.

Hancock, E.W. (2005). Mechanisms of action of newer antibiotics for Gram-positive pathogens. *Lancet Infect Dis.*, 5(4): 209-218.

Harborne, J.B. (1998). Phytochemical Method, a Guide to Modern Technique of Plant Analysis. 3rd Edition Chapman and Hall. New York. pp. 1-198.

Harrison,E.M., Paterson, G.K., Holden,M.T.G., Morgan, F.J.E., Larsen,A.R.,Petersen, A., Leroy, S., De Vliegher, S., Perreten, V., Fox, L.K., Lam, T. J. G. M., Sampimon, O. C., Zadoks, R.N., Peacock, S. J., Parkhill, J. and Holmes, M. A. (2013a). A *Staphylococcus xylosus* isolate with a new *mecC* allotype. *Antimicrob. Agents Chemother*. 57, 1524–1528.

Harrison, E.M., Paterson , G.K.,Holden, M.T. G., Ba, X., Rolo, J., Morgan, F. J. E., Pichon, B., Kearns, A., Zadoks, R. N., Peacock, S. J., Parkhill J., and Holmes, M. A. (2013b). A novel hybrid SCC*mec–mecC* region in*Staphylococcus sciuri*. *Journal of Antimicrobial Chemotherapy*.http://dx.doi.org/ 10.1093/jac/dkt452.

Hashem, F.A., Saleh, M.M. (1999). Antimicrobial components of some cruciferae plants (*Diplotaxis harra Forsk*. and *Erucaria microcarpa* Boiss.). *Phytother. Res*, *13*, 329–332.

Hawkey,P. M. (1998). The origins and molecular basis of antibiotic resistance. *Bmj*317:657–60.

Heyer, G., Saba, S., Adamo, R., Rush, W., Soong, G., Cheung, A. and Prince, A. (2002). *Staphylococcus aureus agr* and *sarA* functions are required for invasive infection but not inflammatory responses in the lung. *Infect. Immun*, 70: 127-133.

Hiramatsu, K. (2004). Elucidation of the mechanism of antibiotic resistance acquisition of methicillin-resistant *Staphylococcus aureus* (MRSA) and determination of its whole geneome nucleotide sequence. *JMAJ*; 47:153–9.

Hollosy, F., Seprodi, J., Orfi, L., Eros, D., Keri, G., Idei, M. (2002). Evaluation of lipophilicity and antitumor activity of parallel carboxamide libraries. *Journal Of Chromatography B-Biomedical Applications* 780:(2) pp. 355-363.

Hostettmann, K. (1999).Strategy for the biological evaluation of plant extracts. *International Union of Pure and Applied Chemistry*, 70, 1109 -1113.

Howland, R.D. and Mycek, M.J.(2006). Lippincott's Illustration Review: Pharmacology. Harvey, R.A, Champe P.C (Eds). Lippincott Williams & Wilkins Publishers London, 157-168.

Huang, C.M., Chen, C.H., Pornpattananangkul, D., Zhang, L., Chan, M., Hsieh, M.F., Zhang, L. (2011). Eradication of drug resistant *Staphylococcus aureus* by liposomal oleic acids. *Biomaterials*, *32*, 214–221.

Hugo WB, Russell AD (1998). Pharmaceutical Microbiology. (6th Edition). Oxford. pp. 91-100.

Ijeh, I.I and Ukweni, A. I. (2007). Acute Effect of Administration of Ethanol Extracts of *Ficus exasperata* Vahl on kidney function in albino rats. In: *Journal of Medicinal Plant Research* 1(2): 027 -029.

ILSI (2005). Flavonoids and Heart Health. In: Proceedings of the ILSI North America Flavonoids Workshop, June, 2005, Washington (USA):34-46.

Inglis, B., Matthews, P.R., Stewart, P.R., (1988). The expression in Staphylococcus aureus of cloned DNA encoding methicillin resistance. *J Gen Microbiol*. 134:1465-1469.

Irvine, F.R. (1961). Woody Plants of Ghana. Oxford University Press, London, 129-130.

Islam, A., Sayeed, A., Bhuiyan, M.S., and Mojaddik, M.A. (2001).Antimicrobial Activity and Cytotoxicity of *zanthoxylum budrunga*. *Fitoterapia 72: 428-430.*

Ito, T., Hiramatsu, K., Tomasz, A., de Lencastre, H., Perreten, V., Holden,M. T. G., Coleman,D. C., Goering, R., Giffard, P.M., Skov,R. L., Zhang,K., Westh, H., O'Brien,F., Tenover, F. C., Oliveira, D. C., Boyle-Vavra, S., Laurent, F., . Kearns, A. M., Kreiswirth, B., Ko, K. S., Grundmann,H.,Sollid, J. E., John Jr,J. F., Daum, R., Soderquist, B., and Buist, G. (2012). Guidelines for reporting novel *mecA* gene homologues. *Antimicrob. Agents Chemother.* 56, 4997–4999.

Ito, T., Katayama, Y., Asada, K., Mori, N., Tsutsumimoto, K., Tiensasitorn, C., Hiramatsu, K.(2001). Structural comparison of three types of staphylococcal cassette chromosome mec integrated in the chromosome in methicillin-resistant *Staphylococcus aureus. Antimicrob Agents Chemother* 45:1323–36.

Ito, T., Ma, X.X., Takeuchi,F., Okuma, K., Yuzawa, H., and Hiramatsu,K.(2004).Novel type V staphylococcal cassette chromosome *mec* driven by a novel cassette chromosome recombinase, *ccrC.* Antimicrob Agents Chemother 48:2637–51.

Iwu, M.W., Duncan, A.R., and Okunji, C.O. (1999). New antimicrobials of plants origin. In: Janick J (ed.), Perspective on new crops and new uses. ASHS Press, Alexandria, VA. pp: 457-462.

Jayeola, A.A. (1998). Surface Sculpturing of *Alstonia boonei* De wild. and *A. congensis* Engl. (Apocyanaceae) and its importance on their taxonomy. *Feddes Report. 109 (5-6):* 429-433.

Jork, H., Funk, W., Fishcer, W., Wimmer, H., and Wiley, N.Y. (1990). Thin Layer Chromatography, 2nd edition, E. Stahl, ed, Springer-Verlag, NY, 1969 TLC Reagents & Detection Methods – Physical & Chemical Detection Methods: Fundamentals, Reagents,I, Vol 1a,

Katayama, Y., Ito, T., Hiramatsu, K. (2000). A new class of genetic element, staphylococcus cassette chromosome *mec*, encodes methicillin resistance in *Staphylococcus aureus. Antimicrob Agents Chemother,*44:1549–55.

Katzung B.G. (1998). Basic and Clinical Pharmacology. *Appleton and Lange, New York*. 682-763.

Katzung, B.G and Parmely, W.W. (1989). Cardiac Glycosides and other drugs use in congestive heart failure. In: Basic and Clinical Pharmacology.Katzung, B.G (ed) 4th edition, Prentice Hill international Inc. Pp. 165-182.

Kechrid, A., Pe´rez-Va´ zquez, M., Smaoui, H., Hariga, D., Rodrı´guez- Ban˜ os, M., Vindel, A., Baquero, F., Canto´ n, R. and Del Campo, R. (2011). Molecular analysis of community acquired methicillin-susceptible and resistant Staphylococcus aureus isolates recovered from bacteraemic and osteomyelitis infections in children from Tunisia. *Clin Microbiol Infect*, 17, 1020–1026.

Kerharo, J and Adam, J.G. (1963). Deuxième inventaire desplantes médicinales et toxiques de la Casamance(Sénégal), *Ann. Pharm. Franc*; 21:853-70.

Kesah, C., Ben-Redjeb, S., Odugbemi, T.O., Boye, C.S.B., Dosso, M., Ndinya-Achola, J.O., Koulla-Shiro, S., Benbachir, M., Rahal, K., Borg, M. (2003). Prevalence of methicillin resistant *Staphylococcus aureus in* eight African hospitals and Malta. *Clin. Microb. Infect.* 9(2), 153-156.

Khatune, N.A., Modsnaddik, M.A and Haque, M.E. (2001). Antibacterial activity and cytotoxicity of *Nyctanthes arbar* tristis flowers. Fitoterapia 72:412-414.

Kim, C., Milheiriço, C.,Gardete, S., Holmes, M.A., Holden[I], M. T. G., de Lencastre,H., and Tomasz,A. (2012). Properties of a novel PBP2A protein homolog from Staphylococcus aureus strain LGA251 and its contribution to the betalactam- resistant phenotype. *J. Biol. Chem.* 287, 36854–36863.

Klein, E., Smith, D.L., and Laxminarayan, R.(2007) Hospitalizations and deaths caused by methicillin-resistant Staphylococcus aureus, United States, 1999–2005. *Emerg Infect Dis* 13: 1840–1846.

Klevens, R.M., Edward, J.R., Tenover, F.C., McDonald, L.C., Horan, T., and Gaynes, R. (2006). Changes in the epidemiology of methicillin resistance *Staphylococcus aureus* in intensive care units in US hospitals, 1992-2003. *Clin. Infect. Dis.* 42, 389-391.

Koch, A. L. (2003). Bacterial wall as target for attack: past, present, and future research. *Clin. Microbiol. Rev.*16:673–687.

Kokate, C.K. (1986). Practical Pharmacognosy 4th ed. Vallabh Prakashan, Delhi – 110088, 2001, pp 115 – 121.

Kong, H.Z. and Liang, Z.Q.(2003). A new *Penicillium* species isolated from Jiangxi, China. Mycostema, 22, 4-5.

Konkwara, J.O. (1976). Medicinal Plants of East Africa. *Literature Bureau*, Nairobi. Pp3-8.

Kumar, R. and Singh, M. (1984). Tannins: Their Adverse Role in Ruminant Nutrition *J. Agric food Chem*. 32: 447 – 453.

Leboffe, J. M., and Pierce, E. B. (1999). Photographic Atlas fot the Microbiology Laboratory. 2nd edition. Pp 85-90

Lewbart, M.L., Wehrli, W., Reichstein, T. (1963). Helv. Chim. Acta 46, 565,in Gauthu. (2008). Dyeing Reagents for TLC-1, https://www.scribd.com/doc/8064894/Dyeing-Reagents-for-TLC-1

Lewington, A., Parker, E and Corner, E.J. (1999).Ancient Trees: Trees that live for 100 years, London, Collins and Brown Ltd, 192p.

Lewis, W.H., Elvin-Lewis, M.P.F. (1977). Medical Botany - plants affecting man's health. John Wiley & Sons.

Litvin, A.A., Kolyvanov, G.B., Zherdev, V.P., Arzamastsev, A.P. (2004).Relationship between physicochemical characteristics and pharmacokinetic parameters of 1,4-benzodiazepine derivatives.*Pharmaceutical Chemistry Journal*, 38 (11), pp. 583-586.

Loffler, C. A., and Macdougall, C. (2007). Update on prevalence and treatment of methicillin-resistant *Staphylococcus aureus* infections. Expert Rev. *Anti-Infect. Ther.*5:961–981.

Lorke, D. (1983). A New Approach to Practical Acute Toxicity testing. Arch Toxicol 1988, 53: 275 -89.

Ma, X.X., Ito, C., Tiensasitorn, C., Jamklang,M., Chongtrakool, P., Boyle-Vavra, S., Daum, R. S., andHiramatsu, K.(2002). Novel type of staphylococcalcassette chromosome *mec* identified in community-acquired methicillin-resistant *Staphylococcus aureus* strains. *Antimicrob Agents Chemother* 46:1147–52.

Malachowa, N and DeLeo, F.R. (2010). Mobile genetic elements of *Staphylococcus aureus.* Cell Mol Life Sci 67:3057.

Mann, A., Gbate, M. and Nda-Umar, A. (2003). Medicinal and Economic Plants for Nupeland. Jube-Evans Publishers.Bida, Niger State, Nigeria pp: 67.

Mann, A., Amupitan, J.O., Oyewale, A.O., Okogun, J.I. and Ibrahim, K. (2007a). An Ethnobotanical survey of indigenous flora for treating tuberculosis and other respiratory diseases in Niger State, Nigeria. *J. Phytomed. & Therap,* 12, 1-12.

Mann, A., Amupitan, J.O., Oyewale, A.O., Okogun, J.I. and Ibrahim, K. (2007b). Antimicrobial potentials of *Anogeissus leiocarpus* and *Terminalia avicennioides* in treating infectious diseases prevalent in Hospital Environments in Nigeria. *J. Women Tech. Edu. & Empl.* 5, 67-74.

Mann, A., Yahaya, Y., Banso, A., and John, F. (2008a). Photochemical and antimicrobial activity of *T.avicennioindes* extracts against some bacterial pathogens associated with patients suffering from complicated respiratory tract diseases. *J Medicinal Plant Research*, 2:94-97.

Mann, A., Amupitan, J.O., Oyewale, A.O., Okogun, J.I., Ibrahim, K., Oladosu, P., Lawson, L., Olajide, I., and Nnamdi, A.(2008b). Evaluation of in vitro antimycobacterial activity of Nigerian plants used for treatment of respiratory diseases. *Afr. J. Biotech.* 7, 1630-1636.

Mann, A., Yahaya, A.Y., Banso, A., and John, F., (2008c). Phytochemical and Antimicrobial activity of *Terminalia avicennioides* extracts against some bacteria pathogens associated with patients suffering from complicated respiratory tract diseases. *J. Med. Plant Res*. 2, 094-097.

Mann, A., Amupitan, J. O., Oyewale, A. O., Okogun, J. I., and Ibrahim, K. (2009).Antibacterial activity of terpenoidal fractions from*Anogeissus leiocarpus* and *Terminalia avicennioides*against community acquired infections..*African Journal of Pharmacy and Pharmacology*. 3(1). pp. 022-025.

Mann, A., Ajiboso, O.S.O., Ajeigbe, S., Gbate, M., and Isaiah, S. (2011). Evaluation of the wound healing activity of ethanol extract of *Terminalia avicennioides* root bark on two wound models in rats. *Inter J Med Arom Plants.*Vol. 2: 1:95-100.

Marino, M., Bersani, C., Comi, G. (2001). Impedance measurements to study the antimicrobial activity of essential oils from lamiaceae and compositae. *Int. J. Food Microbiol.67*, 187–195.

Mbata, T.I and Saika. A. (2008). Antibacterial activity and phytochemical screening of crude ethanolic extract of leaves of *Ocimum gratissimum* L. on *Listeria monocytogenes*.*The internet journal of microbiology*, 4(2): 1-13

Meenaa, V., Gandhiraja, N., Sriram, S., Srilakasmi, J.K., Sasikumar, C. and Rajeswari, R. (2009). Phytochemical screening and antimicrobial activity of the plant extracts of *Mimosa pudica* L. against selected microbes. In: *Ethnobotanical leaflet, 13: 618-24.*

Meroueh, S. O., Bencze, K. Z., Hesek, D., Lee, M., Fisher, J. F., Stemmler,T. L. and Mobashery, S. (2006). Three-dimensional structure of thebacterial cell wall peptidoglycan. *Proc. Natl. Acad. Sci. USA* 103:4404–4409.

Michalec, C., (1956). Biochim. et biophys. Acta 19, 187.

Moreillon, P., Que, Y.A., and Glauser, M.P. (2005). *Staphylococcus aureus* (including staphylococcal toxic shock). In: Mandell, G.L., Bennett, J.E., Dolin, R., eds. *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases*. Vol 2. 6th ed. Philadelphia, Pa: Elsevier; 2321-2351.

Naimi, T.S., LeDell, K.H., Como-Sabetti, K.,Borchardt, S. M., Boxrud, D. J., Etienne, J. S., Johnson, K., Vandenesch, F. Fridkin, S. O'Boyle, C., Danila, R. N., Lynfield, R.(2003).Comparison of community- and health care–associated methicillin-resistant *Staphylococcusaureus* infection. *Journal of American Nedical Association,* 290:2976–84.

Nair, R., Kalariya, T., Chanda, S. (2005). Antibacterial activity of some selected Indian medicinal flora. *Turk J. Biol.* 29: 41-47.

Nascimento, G.G.F., Locatelli, J., Freitas, P.C. and Silva, G.L. (2000). Antibacterial activity of plant extracts and phytochemicals on antibiotic resistant bacteria. *Braz. J. Microbiol, 31,247*-256.

Nina, R., Weiblen, G.D and Cook, J.M. (2005). 60 Million years of Co-divergence in the fig-wasp symbiosis‖. Proceeding of the Royal Society of London Series B, *Biological sciences 272 (1581): 2593-2599.*

O'Hara, F.P., Guex, N., Word, J.M., Miller, L.A., Becker, J.A., Walsh, S.L., Scangarella, N.E., West, J.M., Shawar, R.M., and Amrine-Madsen, H. (2008). A geographic variant of the Staphylococcus aureus Panton-Valentine leukocidin toxin and the origin of community-associated methicillin-resistant S. aureus. *J Infect Dis,* 197:187–94.

Obi, V.I and Onuoha, C. (2000). Extraction and Characterization of Natural Products. In: Biological and Agricultural Techniques, 55-68.

Okuma, K., Iakawa, K., Turnidge, J.D., Grubb,W. B., Bell,J. M., O'Brien,F. G., Coombs,G.W., Pearman,J.W., Tenover,F.C., Kapi,M., Tiensasitorn,C.,Ito,T., and Hir amatsu, K. (2002). Dissemination of new methicillin-resistant *Staphylococcus aureus* clones in the community. *J Clin Microbiol,* 40:4289–94.

Oliveira, D.C., Tomasz, A., and de Lencastre, H. (2002). Secrets of success of a human pathogen: molecular evolution of pandemic clones of methicillin-resistant *Staphylococcus aureus*. *The Lancet Infectious Diseases.* 2:180-189.

Ololade Z.S., Olatunde O.Z., Oyelese O.J., Olaoye O.O., Odewande R.A.(2014). Total Phenolic Content, Free Radical Inhibition, Antioxidant and Antibacterial Potentials of the Medicinal Organic Compounds in the Fruit of *Terminalia catappa* Linn. *Nat Sci*; 12(2):46-50

Olsen, R.J., Burns, K.M., Chen, L., Kreiswirth, B.N., and Musser, J.M. (2008). Severe necrotizing fasciitis in a human immunodeficiency virus-positive patient caused by methicillin-resistant Staphylococcus aureus. *J Clin Microbiol* 46: 1144–7.

Onanuga, A., Oyi A. R., and Onaolapo, J. A. (2005). Prevalence and susceptibility pattern of methicillinresistant *Staphylococcus aureus* isolates among healthy women in Zaria. *Nigeria African Journal of Biotechnology*, Vol. 4 (11), pp. 1321-1324.

Orji, J.O., Alo, M.N., Anyim, C., Okonkwo, E.C. (2012). Antibacterial Activities of Crude Leaf and Bark Extracts of "Icheku" Dialium guineense on Bacterial Isolates from Bronchitis Patients, *IOSR J Pharm Biol Sci*. 1: 21-25.

Ozdemir,G., Karabay, N.U., Dalay, M.C., and Pazarbasi, B. (2004). Antibac-terial activity of volatile component and various extracts of *Spirulina platensis*. *Phytother. Res.* 18:754-757.

Pan, E.S., Charlesbois, E.D., Auerswald, C. (2003). Homeless youth at increased risk for methicillin-resistant *Staphylococcus aureus* (MRSA) colonization [abstract 255]. In: *Program and abstracts of the 41st Annual Meeting of the Infectious Disease Society of America. Alexandria, VA: Infectious Diseases Society of America.*

Panlilio, A.L., Culver, D.H., Gaynes, R.P., Banerjee, S., Henderson, T.S., Tolson, J.S., and Martone, W.J. (1992). Methicillin-resistant *Staphylococcus aureus* in U.S. hospitals, 1975–1991. *Infection Control and Hospital Epidemiology.*13:582-586.

Parfene, G., Hornicar, V., Tyagi, A.K., Malik, A., Bahrim, G. (2013). Production of medium chain saturated fatty acids with enhanced antimicrobial activity from crude coconut fat by solid state cultivation of yarrowia lipolytica. *Food Chem*. 136(3-4):1345-1349.

Paterson, G. K., Harrison, E. M., and Holmes, M. A. (2014). The emergence of mecC methicillin-resistant Staphylococcus aureus, *Trends in Microbiology*, January Review, Vol. 22, No. 1.

Pauk, K., Zadrazilova, I., Imramovsky A. (2013). "New derivatives of salicylamides: preparation and antimicrobial activity againstvarious bacterial species," *Bioorganic and Medicinal Chemistry*, vol. 21, no. 21, pp. 6574–6581, 2013.

Pearman, J.W., and Grubb, W.B. (1993).Emerging strains of multiresistant methicillin-resistant *Staphylococcus aureus* threaten success of screening policy. *APUA Newsletter,* 11:1–8.

Perdreau-Remington, F., Carleton, H.A., Etorma, J., Diep, B. A., Sensabaugh, G. F.(2004). Alarming prevalence of community-acquired methicillin resistant *Staphylococcus*

aureus (CA-MRSA) in hospitalized patients [abstract K-1859a]. In: Program and abstracts of the 44th Annual Interscience Conference on Antimicrobial Agents and Chemotherapy.Washington, DC: American Society for Microbiology.

Pinho, M. G., de Lencastre, H., and Tomasz, A. (2001). An acquired and a native penicillin-binding protein cooperate in building the cell wall of drugresistant staphylococci. *Proc. Natl. Acad. Sci. USA* 98:10886–10891.

Prescot, L.M., Harley, J.P., Klein, D.A. (2002). Microbiology. 5th edition. McGraw-Hill Companies, Inc. New York. p. 811.

Prozesky E.A., Meyer J.J.M. and Louw A.I. (2001).In-vitro antiplasmodial activity and cytotoxicity of ethnobotanically selected South African plants. Journal of Ethnopharmacology, 76, 239-245.

Pulicherla, K.K., Gadupudi, G.S., Rekha, V.P.B., Seetharam, K., Kumar, A., and Sambasiva Rao, K.R.S. (2011). Isolation, Cloning and Expression of Mature Staphylokinase from Lysogenic *Staphylococcus aureus* Collected from a Local Wound Sample in a Salt Inducible *E.coli* Expression Host. *International Journal of Advanced Science and Technology,* 30, 35-42.

Quave, C.L., Plano, L.R.W., Pantuso, T., and Bennett, B.C., (2008). Effects of extracts from Italian medicinal plants on planktonic growth, biofilm formation and adherence of methicillin-resistant *Staphylococcus aureus. Journal of Ethnopharmacology*. 118:418-428.

Richards, M. J., Edwards, J. R., Culver, D. H., Gaynes, R. P. (1999a). Nosocomial infections in pediatric intensive care units in the United States. *Pediatrics*103:e39.

Richards, M. J., Edwards, J. R., Culver, D. H., Gaynes, R. P. (1999b). Nosocomial infections in medical intensive care units in the United States. *Crit. Care Med.*27:887–892.

Roberts, R. B., Tennenberg, A. M., Eisner, W., Hargrave, J., Drusin, L. M., Yurt, R., Kreiswirth, B. N.(1998). Outbreak in a New York City teaching hospital burn center caused by the Iberian epidemic clone of MRSA. *Microb Drug Resist,* 4:175–83.

Robinson, D.A., and Enright, M.C. (2004). Multilocus sequence typing and the evolution of methicillin-resistant *Staphylococcus aureus. Clin Microbiol Infect,* 10:92–7.

Rybak, M., Lomaestro, B., Rotschafer, J.C., Moellering, R., Craig, W., Billeter, M., Dalovisio, J.R., Levine, D.P. (2009). Therapeutic monitoring of vancomycin in adult patients: A consensus review of the American Society of Health-System Pharmacists, the Infectious Diseases Society of America, and the Society of Infectious Diseases Pharmacists. *Am. J. Health Syst. Pharm.* 2009, *66,* 82–98.

Safdar, N., Fox, B.C., and McKinley, L.M. (2007). Epidemiology of MRSA. In: Weigelt JA, ed. *MRSA. Informa Healthcare USA*; 11-30.

Samy R.P., Ignacimuthu S. and Sen A. (1998).Screening of 34 Indian medicinal plants for bacterial properties. *Journal of Ethnopharmacology*, 62, 173-181.

Sanford, M.D., Widme, A.F., Bale, M.J., Jones, R.N., and Wenzel, R.P. (1994). Efficient detection and long-term persistence of the carriage of methicillin-resistant Staphylococcus aureus. *Clin Infect Dis*, 19:1123-1128.

Sanon, S., Gansane, A., Ouattara, L.P., Traore, A.A., Ouedraogo, I.N., Tiono, A, Donatella,T., Nicoletta, B., Sirima,S. B. (2013). *In vitro*antiplasmodial and cytotoxic properties of some medicinal plants from Western Burkina Faso. *Afr J Lab Med*, 81:1-7.

Scherbanvoski, J.C. (1971). Rast Rtesus Nikitskii Bot Sad USSR, 17:133- 141.

Shai, A., Natalya, M.D., Bilenko, M.D., Zeev, R.B. and Halevy, M.D. (2004). Use of infection control procedures in an out-patient clinic for leg ulcers and the rate of contamination with methicillin-resistant *Staphylococcus aureus. Wounds*, 16(6):15-25.

Shokeen, P., Bala, M. and Tondon, V., (2009). Evaluation of the activity of 16 medicinal plants against *Neisseria gonorrhea. Int. J. Antimicrob. Agents*., 33: 86-91.

Shore, A. C., Deasy,E. C., Slickers, P., Brennan, G., O'Connell, B., Monecke, S., Ehricht, R. and Coleman,D., C. (2011). Detection of staphylococcal cassette chromosome mec type XI carrying highly divergent *mecA, mecI, mecR1, blaZ,* and *ccr* genes in human clinical isolates of clonal complex 130 methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother*. 55, 3765–3773.

Soladoye, M. O., and Chukwuma, E. C., (2012). Quantitative phytochemical profile of the leaves of *Cissus populnea* Guill. & Perr. (Vitaceae) – an important medicinal plant in central Nigeria. *Scholars Research LibraryArchives of Applied Science Research*, 4 (1):200-206.

Song, M.D., Wachi, M., Doi, M.,Ishino, F., Matsuhashi, M.(1987). Evolution of an inducible penicillintarget protein in methicillin-resistant *Staphylococcus aureus* by gene fusion. *FEBS Lett* 221:167–71.

Sreevidya, N., and Mehrotra, S. (2003). Spectrophotometric method for estimation of alkaloids precipitable with dragendorff's reagent in plantmaterials, *journal of aoac international*, 8: 6

Stapleton, P.D., and Taylor, P.W. (2002). Methicillin resistance in Staphylococcus aureus: mechanisms and modulation. Sci Prog 85: 57–72.

Stephen, T. O., and Kennedy, K. A. (2011). Bacteria Resistance to Antibiotics: Recent Trends and Challenges. Int J Biol Med Res, 2(4): 1204 - 1210

Stevens, D. (2007). Genetics of MRSA: The United States and Worldwide. In: Weigelt JA, ed. *MRSA.* Informa Healthcare USA; 31-42.

Sutherland, R., and Rolinson, G.N. (1964). Characteristics of methicillin-resistant staphylococci. *J Bacteriol* 87:887–99.

Swain, T. (1979). Tannins and Lignins. In: Herbivores: Their Interactions with Plant Metabolites. Rosenthal, G.A., and Janzen, D.H., (Eds.). Academic Press, New York.

Tagboto, S. and Townson, S. (2001). Antiparasitic properties of medicinal plants and other naturally occurring products. *Adv. Parasitol.*,50: 199-295.

Takeda, K., Hara, S., Wada, A., Matsumoto, N., Chromatog, J. (1963). 11, 562in Gauthu. (2008). Dyeing Reagents for TLC-1, https://www.scribd.com/doc/8064894/Dyeing-Reagents-for-TLC-1

Tiemersma, E.W., Bronzwaer, S.L., Lyytikainen, O., Degener, J.E., Schrijnemakers, P., Bruinsma, N., Monen, J., Witte, W., and Grundman, H. (2004). European Antimicrobial Resistance Surveillance System Participants. Methicillin-resistant Staphylococcus aureus in Europe, 1999–2002. *Emerg Infect Dis* 10: 1627–1634.

Tipparaju K. S.,Mulhearn, C. D.,Klein M.G. , Chen,Y., Tapadar,S., Bishop, H. M., Yang,S., Chen,J., Ghassemi,M., Santarsiero, D. B. , Cook, L.J. , Johlfs,M., Mesecar, D.A., Johnson, E. M.,Kozikowski, P. A. (2008).Design and Synthesis of Aryl Ether Inhibitors of the *Bacillus Anthracis* Enoyl–ACP Reductase.*ChemMedChem*. 3(8): 1250–1268.

Tomasz, A., Nachman, S., and Leaf, H. (1999). Stable classes of phenotypic expression in methicillin-resistant clinical isolates of staphylococci. *Antimicrob Agents Chemother*, 35:124–9.

Trease, G.E., and Evans,W.C. (1989). *Pharmacognosy*. 13th (ed). ELBS/Baillier Tindall, London. 345-6,535-6,772-3

Tscheche, R. J., (1961). Chromatog. 5:217 in Gauthu. (2008). Dyeing Reagents for TLC-1, https://www.scribd.com/doc/8064894/Dyeing-Reagents-for-TLC-1.

Tschesche, R. Grimmer, G. and Seehofer, F. (1953). U¨ ber pflanzliche Herzgifte, XXIV. Mitteil.: Die quantitative Trennung undIdentifizierung von Herzgiftglykosiden aus Digitalis purpurea und lanatadurch “echte Verteilungschromatographie anPapier”, *Chem Ber* 86:1235–1241.

Tsubakishita, S., Kuwahara-Arai, K., Baba, T. and Hiramatsu, K. (2010). Staphylococcal cassette chromosome meclike element in Macrococcus caseolyticus. *Antimicrob. Agents Chemother.* 54, 1469–1475.

Udo, E.E., Pearman, J.W., and Grubb, W.B. (1994). Emergence of high-level mupirocin resistance in methicillin-resistant *Staphylococcus aureus* in Western Australia. *J Hosp Infect,* 26:157–65.

Ukwubile, C. A., Otalu, O. Jr., lamidi, T. B., and Iganga, E. M. (2013).Antibiotic Resistance Mechanisms of Impetigo Contagiosa Bacteria (Staphylococcus aureus and Streptococcus pyogenes) in Infants and the Efficacy of Bridelia ferruginea Benth. (Phyllanthaceae) and Terminalia avicennioides Guill. & Perr. (Combretaceae) Ethanol Extracts.*IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)*, Vol. 7PP 08-18.

Van Wyk, B., and Wink, M. (2004). Health disorders and Medicinal plants. In: Medicinal plants of the world. Published by Briza Publications CK90/11690/23, P O Box 56569, Arcadia 0007, Pretoria, South Africa, Pp 351-370.

Vollmer, W., Blanot, D. and de Pedro, M. A. (2008). Peptidoglycan structure and architecture. *FEMS Microbiol.* Rev. 32:149–167.

Von Maydell H.J., (1996).Trees and Shrubs of the Sahel. Verlag Josef Margnaf, Weikersheim, 562.

Waxman, D. J., and Strominger, J. L. (1983). Penicillin-binding proteins and the mechanism of action of beta-lactam antibiotics. *Annu. Rev. Biochem.*52:825–869.

Wisplinghoff, H., Rosato, A. E., Enright, M.C., Noto, M.,Craig,W.,Archer,G. L.(2003). Related clones containing SCC*mec* type IV predominate among clinically significant *Staphylococcus epidermidis* isolates. *Antimicrob Agents Chemother* 47:3574–9.

World Health Organization. (2002). Antimicrobial resistance fact sheet. pp: 94.

Wu, S.W., DE Lencastre, H., and Tomasz, A. (2001). Recruitment of the *mecA* gene homologue of *Staphylococcus sciuri* into a resistance determinant and expression of the resistant phenotype in *Staphylococcus aureus. J Bacteriol* 183:2417–24.

Wyatt, E. E., Galloway R. J. D. W., Thomas L. G.,Welch M., Loiseleur, O., Plowrightd, T. A., and Spring, D. R. (2008) Identification of an anti-MRSA dihydrofolate reductase inhibitor from a diversity-oriented synthesis. *Chem. Commun*., 4962–4964.

Zadrazilova, I., Pospisilova, S., Pauk, K., Imramovsky, A., Vinsova, J., Cizek, A., Jampilek, J. (2014). *In Vitro* Bactericidal Activity of 4- and 5-Chloro-2-hydroxy-*N*-[1-oxo-1-(phenylamino)alkan-2-yl]benzamides against MRSA

Zapun, A., Contreras-Martel, C., and Vernet, T. (2008). Penicillin-binding proteins and beta-lactam resistance. *FEMS Microbiol.* Rev. 32:361–385.

Zhang, H.Z., Hackbarth, C.J., Chansky, K.M., and Chambers, H.F. (2001). A proteolytic transmembrane signaling pathway and resistance to beta-lactams in staphylococci. *Science* 291: 1962–5.

Zhang, R., Eggleston, K., Rotimi, V., and Zeckhauser, R.J. (2006). Antibiotic resistance as a global threat: Evidence from China, Kuwait and the United States. *Globalization and Health* 2(6), 1-14.

APPENDICES

APPENDIX 1.0

VERIFICATION OF RESISTANCE OF ATCC 33591 STRAIN TO METHICILLIN/OXACILLINE ANTIBIOTIC USING 1µg OXACILLIN ANTIBIOTIC DISK AS DESCRIBED IN THE PERFORMANCE STANDARD FOR ANTIMICROBIAL SUSCEPTIBILITY TESTING AS DESCRIBED IN THE CINICAL AND LABOURATORY STANDARD INSTITUTE 2007.

Chloramphenicol 30µg (**C30**) Streptomycin 10 µg (**S10**)

Penicillin G 10 U (**P10**) Amikacin 30µg (**AK30**)

Trimethoprim 5 µg (**W5**) Oxacillin 1µg (**OX1)**

APPENDIX 2.0

PREPARATION OF STANDARD REAGENTS FOR QUANTITATIVE PHYTOCHEMICAL ESTIMATION AND SPRAY REAGENTS FOR NATURAL PRODUCT TLC VISUALISATION.

Estimation of alkaloids:

(a) *DR*.—Prepared by mixing (*1*) solution of 0.8 g bismuth nitrate pentahydrate in 40 mldistilled water and 10 ml glacial acetic acid, and (*2*) solution of 8.0 g potassium iodide in 20 ml distilled water.

(b) *Standard bismuth nitrate solution*.—Bismuth nitratestock solution was made by dissolving 10 mg Bi $(NO_3)_3 \cdot 5H2O$ in 5 ml concentrated nitric acid and diluting to 100 ml withdistilled water.

(**c**) *Thiourea, 3%*.—prepared by dissolving 3 g in 100ml distilled water.

(**d**) *Disodium sulfide, 1%*.—prepared by dissolving 1 g in 100 ml distilled water.

Estimation of tannins:

(a) Na_2CO_3, 17% — prepared by dissolving 17 g in 100ml distilled water.

Test for Alkaloids (Dragendorff spray reagent test)

Solution 1: 1.7g basic bismuth nitrate and 20g tartaric acid in 80ml water.

*Solution 2:*16 g potassium iodide in 40ml water.

Stock solution (stable for several weeks in a refrigerator): Mix equal volumes of solutions 1 and 2.

Spraying solution: Dissolve 10g tartaric acid in 50ml water and 10ml stock solution.

Test forCardiac glycosides (Spray test)

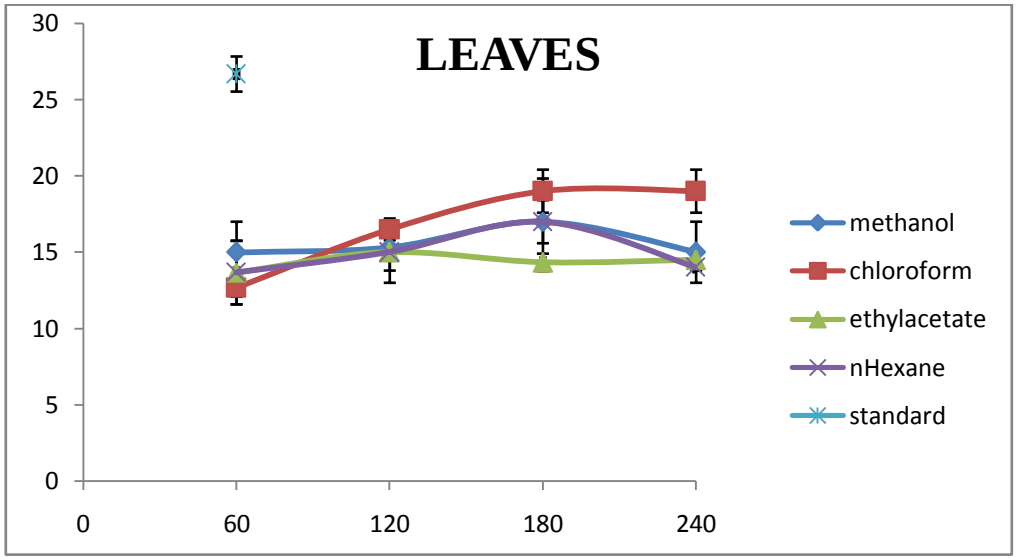
A. Spray solution: 3,5-dinitrobenzoic acid (1 g)was dissolved in a mixture of 50 ml methanol and 50 ml potassium hydroxide solution (c = 2 mol/L).

B. Spray solution I: 2% methanolic solution of 3, 5-dinitrobenzoic acid.

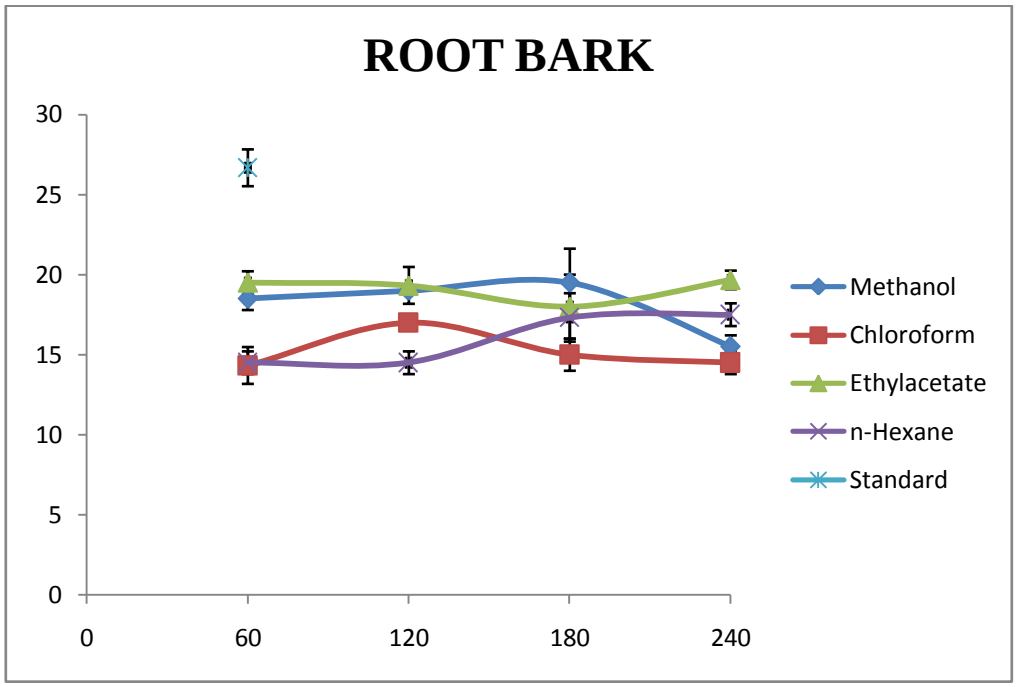
Spray solution II: 5.7% methanolic potassium hydroxide solution.

APPENDIX 3.0

MEAN ZONE IN (MM) OF INHIBITION AT DIFFERENT CONCENTRATIONS OF (60µg/ml, 120µg/ml, 180µg/mlAND 240µg/ml) OF EXTRACTS OF*T. AVICENNIOIDES*LEAF ON MRSA

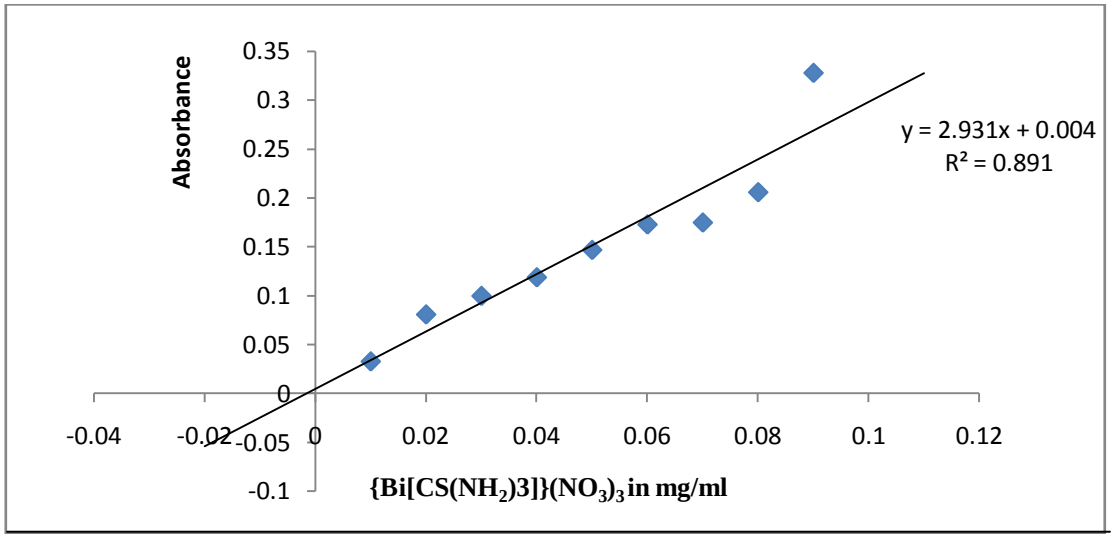


MEAN ZONE IN (mm)OF INHIBITION AT DIFFERENT CONCENTRATIONS OF (60µg/ml, 120µg/ml, 180µg/mlAND 240µg/ml) OF EXTRACTS OF *T. AVICENNIOIDES*ROOT BARK ON MRSA



APPENDIX 4.0

CALIBRATION CURVE FOR QUANTITATIVE ESTIMATION OF ALKALOID IN ETHYL ACETATE ROOTS BARKS EXTRACT OF *TERMINALIAAVICENNIOIDES*USING BISMUTH NITRATE–THIOUREA COMPLEX{$Bi[CS(NH_2)_3]$}$(NO_3)_3$FORMED FROMBISMUTH REACTION WITH THIOUREA IN NITRIC ACID MEDIUM.



(i). At 5mg/ml = 0.5mg/ml
0.5mg/ml + 5ml =0.5mg in 6ml
If 0.5mg in 6ml, therefore
[0.083mg × (12.05 factor)]/ml =

1mg/ml

From Regression Equation,
Y= 2.9317x + 0.0047
If y= 0.215, x=?
0.215=2.9317x + 0.0047
-2.9317x = -0.215 + 0.0047
x = $\frac{-0.215 + 0.0047}{-2.9317}$
x = $\frac{0.2197}{2.9317}$
x = 0.0749395
x=0.075mg

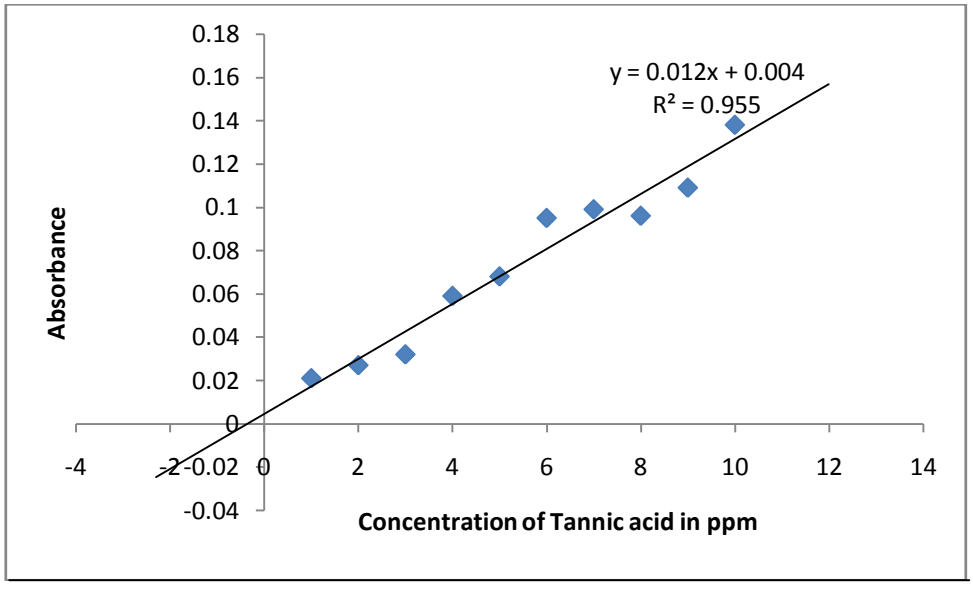
Amount of Bi $(NO_3)_3.5H_20$ = Abs.Value × Dilution Factor (DF),

Where DF =Conc. of sample × Abs.Value
= x × y
= 0.075mg × 0.215
= 0.016125mg
Therefore; Amount of Bi $(NO_3)_3.5H_20$
= 0.215 × 0.016125mg
= 0.00346688
= 0.003mmg × (12.05 factor)]/ml for 1mg/ml
= 0.042mg/ml = 42µg/ml

Because the complex formed is 1:1, the amount of bismuth corresponds to the amount of alkaloids present; hence the method of preparation of Dragendorff's reagent (DR) does not affect the result. The amount of alkaloid present is calculated by multiplying absorbance with factor.

APPENDIX 5.0

CALIBRATION CURVE FOR QUANTITATIV ESTIMATION OF TANNINS IN ETHYL ACETATE ROOTS BARKSEXTRACT OF *Terminaliaavicennioides* USING STANDARD TANNIC ACID



20mg/ml = 2mg/ml = 0.2mg/0.1ml

Therefore, 0.2mg in 0.1ml + 4.9ml add up = 0.2mg in 5ml = 0.04mg/ml
To establish 0.04mg in 1mg, for 1ml, you multiply by (25 factor)
Therefore 0.04mg × (25 factor) = 1mg/ml
From Regression Equation,
Y= 0.0127x + 0.0046
If y = 0.089, x=?
0.089 = 0.0127x + 0.0046

-0.0127x = -0.089 + 0.0046

$$x = \frac{-0.089 + 0.0046}{-2.0127}$$

$$x = \frac{0.0936}{0.0127}$$

x = 7.37007874
Therefore the amounts of Tannins "x" in 1mg/ml of Ethylacetate crude extract
"X" = Conc. x in 0.04mg × (25 factor)
"X" = 7.37007874 × (25 factor)
"X" = 184.25ppm = 0.18425mg/ml = 184.25µg/ml

APPENDIX 6.0

CALULATION FOR QUANTITATIVE ESTIMATION OF ANTRAQUINON IN 0.5mg/ml OF ETHYL ACETATE ROOT BARKS EXTRACT OF *Terminaliaavicennioides* USING ALIZARIN AS STANDARD

10mg ethylacetate crude extract + 10ml of dist. H_20 + 10ml of 50% Methanol
= 10mg in 20ml
= 0.5mg/ml
To convert 0.5mg to 1mg per ml, you multiply by (2 factor)
0.5mg × (2 factor) = 1mg/ml

$$\frac{C_I}{C_2} = \frac{A_1}{A_2}$$

Where C_1 = Concentration of Sample.

C_2 = Concentration of Standard.

A_1 = Absorbance of Sample.

A_2 = Absorbance of Standard.

$$\frac{C_1}{0.02\text{mg/ml}} = \frac{0.169}{0.342}$$

$$C_1 = \frac{0.169}{0.342} \times 0.02\text{mg/ml}$$

$C_1 = 0.49415205 \times 0.02$mg/ml
$C_1 = 0.00988304 \times$ (2 factor)
$= 0.01976608$ mg/ml
$C_1 = 19.80$ µg/ml of Anthraquinone.

Percentage Composition of Anthraquinone Present In 1mg/ml of Ethyl Acetate Roots Barks Extractof *Terminalia avicennioides.*

$$\frac{19.80\mu g}{1000\mu g} \times \frac{100}{1}$$

$= 0.0198 \times 100$

= 1.98% of Anthraquinone per 1mg/ml of ethyl acetate roots barks extract of*Terminalia avicennioides.*

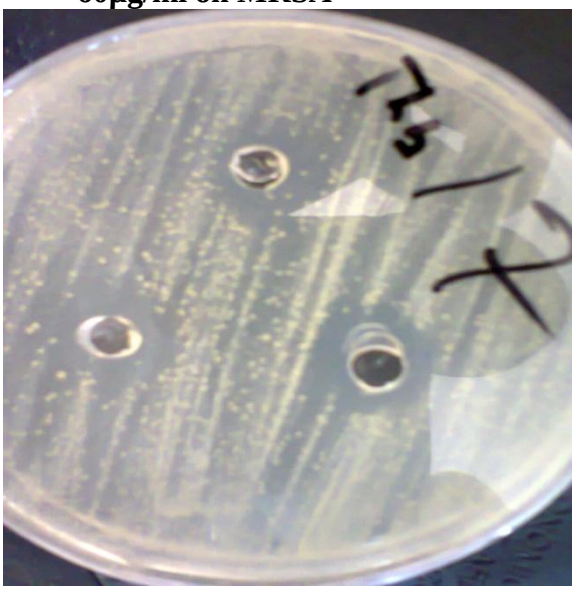
APPENDIX 7.0

ZONE OF INHIBITION OF TLb 4 AND TLb 17 AT DIFFERENT CENTRARATIONS, 60ug/ml AURGUMENTIN (NEGATIVE CONTROL) AND DMSO (POSITIVE CONTROL)ON MRSA

Zone of inhibition of TLb 17 at 120µg/ml on MRSA	**Zone of inhibition of TLb 4 at 120µg/ml on MRSA**

Zone of inhibition of TLb 17 at 60µg/ml on MRSA

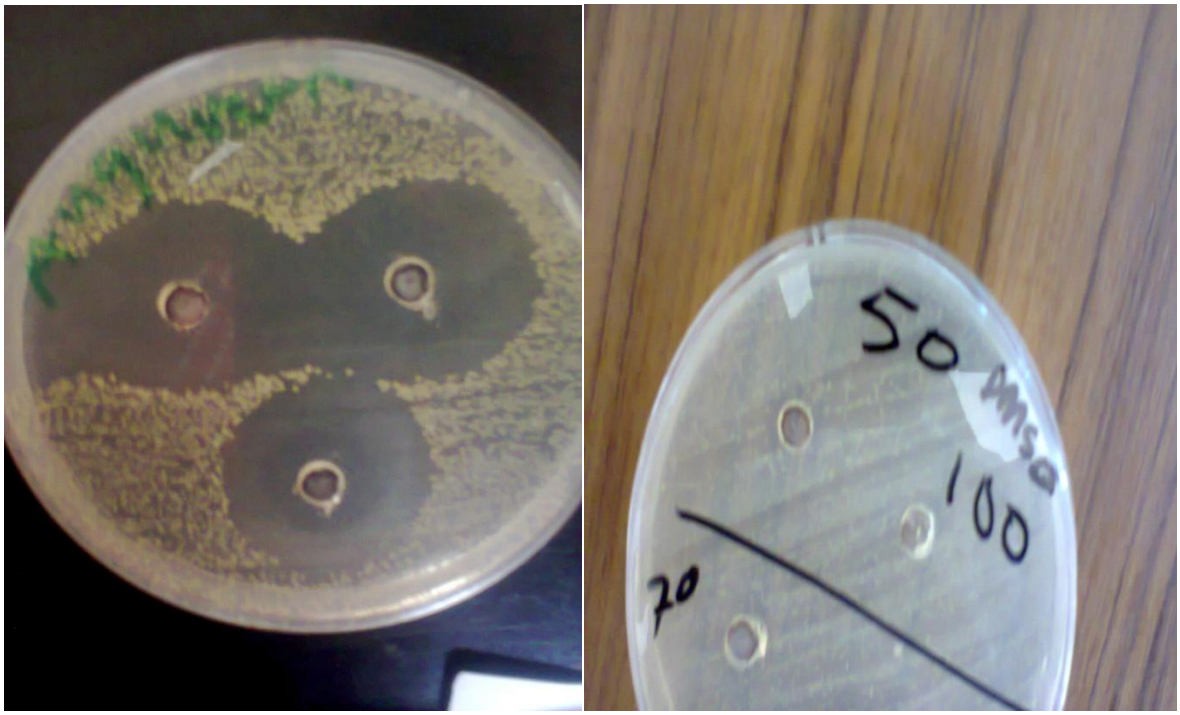
Zone of inhibition of TLb 4 at 60µg/ml on MRSA



APPENDIX 7.0

Zone of inhibition of60ug/ml Aurgumentine On MRSA. (Negative control).

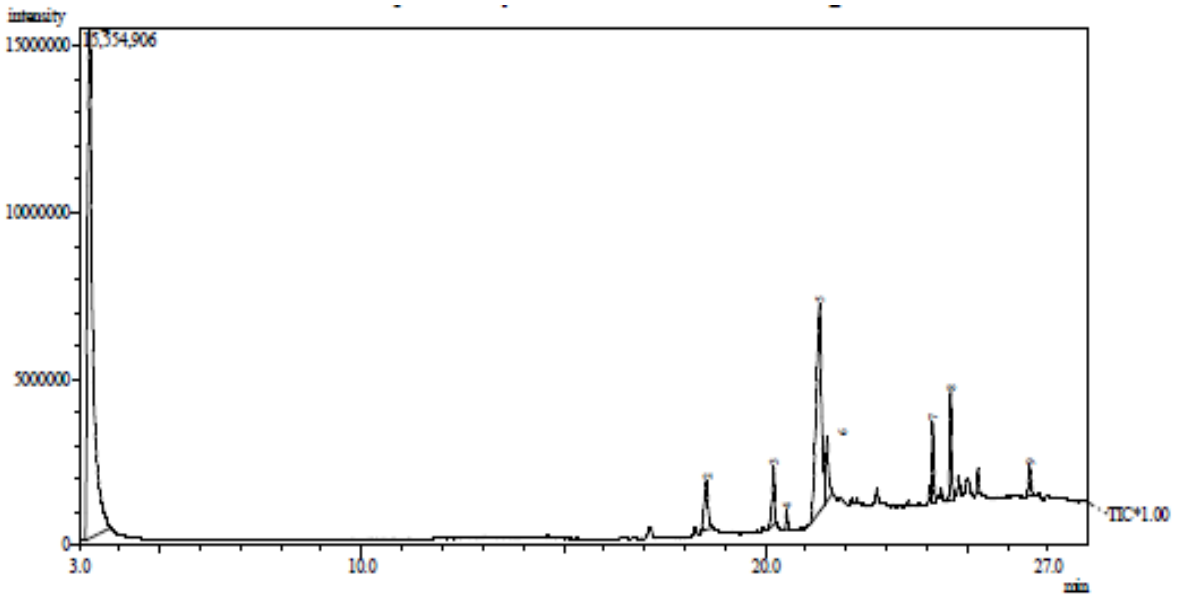
No Zone of Inhibition using DMSO (Reconstitution solvent) on MRSA. (Positive Control).



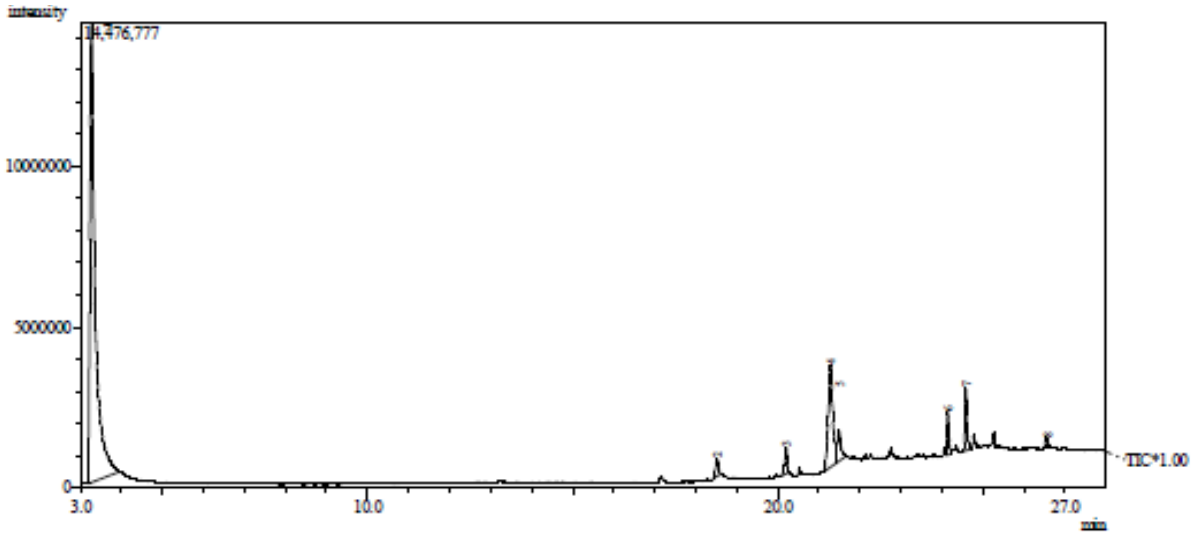
APPENDIX 8.0

THE GAS CHROMATOGRAPHY MASS SPECTROSCOPY SPECTRUM

THE GC-MS SPECTRUM OF FRACTION TLB4 OF ROOT BARKS BIOACTIVE ETHYL ACETATE EXTRACT OF *Terminalia avicennioides*SHOWING FRAGMENTS PEAKS



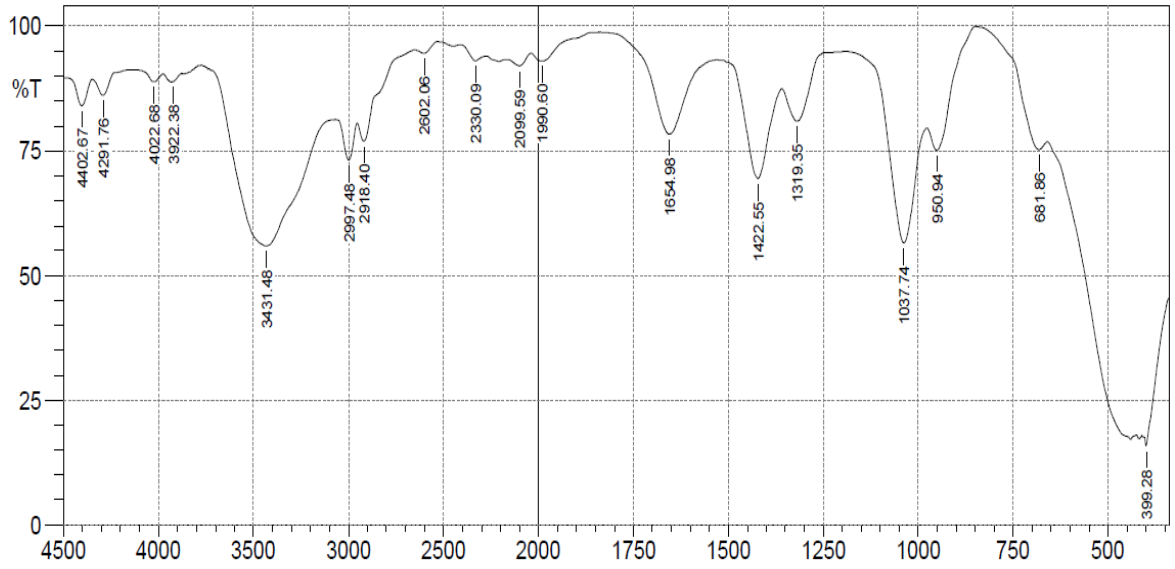
THE GC-MS SPECTRUM OF FRACTION TLB17 OF ROOT BARKS BIOACTIVE ETHYL ACETATE EXTRACT OF *Terminalia avicennioides*SHOWING FRAGMENTS PEAKS



APPENDIX 9.0

FOURIER TRANSFORM INFRA-RED SPECTROSCOPYPECTRUM

THE FTIR SPECTRUM OF FRACTION TLB4 OF ROOT BARKS BIOACTIVE ETHYL ACETATE EXTRACT OF *Terminalia avicennioides*SHOWING SPECTRA PEAKS



THEFTIR SPECTRUM OF FRACTION TLB17 OF ROOT BARKS BIOACTIVE ETHYL ACETATE EXTRACT OF*Terminalia avicennioides*SHOWING SPECTRA PEAKS