EVALUATION OF ANTIBACTERIAL ACTIVITY OF METHANOLIC EXTRACT OF LEAF AND ROOT OF *ZIZIPHUS LOTUS* L ON MULTIDRUG RESISTANT BACTERIA

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SEPTEMBER, 2018

DECLARATION

I declare that the work in this dissertation entitled EVALUATION OF ANTIBACTERIAL ACTIVITY OF METHANOLIC EXTRACT OF LEAF AND ROOT OF *ZIZIPHUS LOTUS L* ON MULTIDRUG RESISTANT BACTERIA has been carried out by me in the Department of Biochemistry, Faculty of life Sciences, under the supervision of Prof. K. M. Anigo, Prof. E. Onyike and Dr. M. S. Aliyu. The information derived from the literature has been dully acknowledged in the text and a list of references provided. No part of this dissertation has previously been presented for another degree or diploma at this or any other Institution.

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CERTIFICATION

This dissertation entitled 'EVALUATION OF ANTIBACTERIAL ACTIVITY OF METHANOL EXTRACT OF LEAF AND ROOT OF *ZIZIPHUS LOTUS* L ON MULTIDRUG RESISTANT BACTERIA by ALBERT DAWAAR DAYO meets the regulations governing the award of the degree of Masters of Science in Biochemistry of the Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

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ABBREVATIONS

ATP	Adenosine triphosphate
CFU	Colony forming unit
CLSI	Clinical laboratory standard Institute
CTX-M	Cefotaxime
DMSO	Dimethyl Sulphoxide
DNA	Deoxy ribonucleic acid
ESBL	Extended Spectrum Beta lactamases
GC-MS	Gas chromatography Mass Spectrometry
LPS	Lipopolysaccharide
MBC	minimum bactericidal concentration
MDR	Multidrug Resistance
MIC	Minimum inhibitory concentration
NCE	Novel chemical entities
PBP	Penicillin binding protein
QRDR	Quinolone resistant determining region
RNA	Ribonucleic acid
SD	Standard Deviation
SHV	Sulhydril Variable
TLC	Thin Layer Chromatography
VRE	Vancomycin resistant enterococci
WHO	World Health Organization

ABSTRACT

Treatment of infectious disease is a major public health challenge due to the continual increase in drug resistance by microorganisms. Plants are rich source of antibacterial agent which could be used to treat and manage human disease.This study was carriedout to evaluate antibacterial activity of methanolic leaf and root extracts of *Ziziphus lotus* on multidrug resistant *E.coli, S. aureus, Salmonella* spp, and *P. aeruginosa*. The Plant materials were extracted with methanol using cold maceration method and concentrated under vaccum with rotary. The crude extracts were fractionated using n-hexane, ethyl acetate and n- butanol. The n- butanol fraction with highest activity was partially purified using silica gel column chromatography. Antibacterial activity of the extract was evaluated using agar well diffusion method at concentrations of 200, 100, 50 and 25mg/ml. The concentrations were prepared by dissolving 2g of each extract in 10ml of 5% dimethyl sulphoxide and the rest were prepared by two fold dilutions. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by broth dilution and spread plate technique respectively. Ciprofloxacin (10µg) was used as standard drug during the study while 5% Dimethyl sulphoxide (DMSO) was used as negative control. All extracts have activity with highest against *Staphylococcus aureus* and least against *Escherichai coli*. Phytochemical screening of the crude extracts revealed the presence of flavonoids, alkaloids, tannins, saponins, cardiac glycosides and cyanogenic glycosides. The GC-MS result showed the presence of Octadec-9-enoic acid, Hexanoic acid undecylester, Floxuridine and Oleic acid in the root and Eicosene, 6- Octadecanoic acid and Quinoline in leaf of partially purified fractions. The MIC value was lowest against *S. aureus* and highest against *E. coli* (12.50mg/ml) and (25mg/ml) respectively.From the finding, *Ziziphus lotus* has significant activity to justify its use in traditional medicine.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background To The Study

Microbial infection is still one of the leading causes of morbidity and mortality especially in developing countries, despite great advancement in antimicrobial therapies and infection control strategies (Barre-Sinoussi 2009; Vargas *et al*., 2003). Throughout the history of human population, microbial infection has been the major cause of disease especially in immune compromised hosts (Senka *et al.,* 2008). The discovery of antibiotics was thought to be the end of the problem however, bacteria have been able to develop mechanism of defense and become resistant to most of the antibiotics (Senka *et al.,* 2008). Drug resistance by pathogenic microorganism has been reported all over the world. Consequently, the increasing prevalence of multidrug resistant strain of microorganism raises an urgent need to search for new sources of antimicrobial agent alongside other strategies such as regulated and rational use of antibiotics (Matu *et al.,* 2012).

Traditional healers have for long been the main providers of primary healthcare using herbal preparation in their practice. Researchers have turned to natural sources to solve the problems of antimicrobial therapies failure. Due to the medicinal value of these natural products they continued to play a dominant role in the maintenance of human health since ancient times. The search on natural product have led to the discovery of novel drug candidates used against diverse diseases, as over 50% of all modern clinical drugs are of such origin (Lawal *et al.,* 2015). A knowledge of the chemical constituents of natural product is desirable not only for the discovery

of therapeutic agents, but also because such information may be of great value in disclosing new sources of bioactive principle for the synthesis of complex chemical substances and discovering the actual significance of folkloric remedies (Lawal *et al*., 2015). Hence a thorough validation of the natural products with medicinal reputation has emerged with emphasis and priority on standardization of natural drugs and products since several bioactive agents have complementary and overlapping mechanism of action (Bashir *et al.*, 2015).

Plants have served as the richest source of raw materials for traditional as well as modern medicine, particularly in Africa and Nigeria (Tsakala *et al*., 2006). The study of many chemical constituents contained in plant is very essential as most drugs used as medicines were synthesized after a careful study of their constituents and structures. However, the main problem is that some drugs are scarce, costly and unavailable to the common man, hence a study of the medicinal importance of plants, and their uses towards curing diseases as a possible solution to development of less costly and effective drugs from local raw materials (Yusuf *et al*.,2014).

Treatment of infections continues to be a problem in modern time because of several side effects of some drugs and growing resistance to antimicrobial agents. Alternative action must be taken to reduce or circumvent the incidence of conventional therapeutic failure to antimicrobial treatment. Among the potential sources of therapeutic agents many plants have long been studied and found to have produced secondary metabolites that protect them against varieties of pathogens (Satish *et al.,* 2008).These compounds have been associated with the used of some plants in folk medicine in the treatment of various diseases (Satish *et al.,* 2008). Evaluation of such plants for various biological activities is important in isolation and characterization of bioactive compound for further development of new drugs (Foutse *et al.*, 2014).

Plant derived drugs remain an important resource especially in developing countries to combat serious diseases. Approximately 60-80% of the world's population still relies on traditional medicines for the treatment of common illnesses (Dev, 2010; Tekeli *et al.,* 2011). Plants produce a wide variety of secondary metabolites many of which have been reported to be of therapeutic value and a promising source of antibacterial compounds, raising hopes of obtaining novel antibiotics that can aid the fight against drug resistant infections (EL-Zawahry *et al.,* 2013)

1.2 Statement of Research Problem.

Bacteria and fungi of clinical importance have developed defense mechanism against antimicrobial agents which made them resistant to both new and old drugs making the drugs not to be effective against the targeted organism (EL-Zawahry *et al.,* 2013)

Most Orthodox drugs are both scarce and expensive to common man and have some side effects on the host. As a result managing patients especially in developing countries is difficult and expensive.

The spread of antimicrobial resistant infections and emergence resistant strain of bacteria are at the increase due to ineffectiveness of antimicrobial drugs used.

Treatment of infections continues to be a problem in modern time because of several side effects of some drugs and growing resistance to antimicrobial agents (Satish *et al.,* 2008).

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1.3 Justification of the Study

About 80% of Nigerian population living in the villages depends solely on herbal medicine for their health care needs (Oladele *et al.,* 2012), therefore the need to research on these plants to prove their efficacy and safety. Plants contain secondary metabolites (phytochemicals) which are potent bioactive compounds against microorganisms and other disease causing pathogens making them useful in folk medicine (Satish *et al.,* 2008). Extracts from various plant parts have been used traditionally to treat ulcer, pulmonary ailment, fever and wounds therefore, it is possible that the plant may have antimicrobial effect (Adzu *et al.,* 2001).

1.4 Aim of the study

The aim of this study is to evaluate antibacterial activities of methanolic extracts of leaf and root of *Ziziphus lotus* on some multidrug resistant bacteria.

1.4.1Specific Objectives of the Study

(i) Carry out qualitative and quantitative determination of phytochemical content of the plant extract.

(ii) Fractionate the crude methanolic extract and evaluate the antibacterial effect of the fractions

(iii) Partially purify the most active fraction and characterize the fraction

(iv) Determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the crude extract and the fraction with highest activity.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Multidrug Resistance

Drug resistance refers to a situation in which the drugs that usually destroy the bacteria no longer do so. Multidrug resistance is a condition enabling a disease causing organism to resist distinct drugs or chemicals of a wide variety of structure and function targeted at eradicating the organism (Iqbal *et al*., 2014). In other word, it implies that people can no longer be effectively treated against the bacteria with those drugs. Antibiotic resistance is now recognized as a major global health security issue that threatens a return to the pre-antibiotic era, with potentially catastrophic economic, social and political ramifications. Resistance has been reported for entire classes of antibiotics, and untreatable multi drug resistant bacteria are increasingly documented (Gemma and Merrett 2013).

The increase in antibiotics resistance has been attributed to a combination of microbial characteristics, use of antibiotics, social and technical changes that enhances the transmission of resistance organisms. This growing threat of resistant organism called for concerted action to prevent the emergence of new resistant strains and the spread of the existing ones (Dzidic *et al.*, 2008). The nutritive and therapeutics antibiotics treatment of farm animal amount to half of the world antibiotics output and has also resulted in antibiotic resistant bacteria. This is because antibiotics pass through the food chain to man (Dzidic *et al*., 2008).

2.1.1 Causes of drug resistance

Inappropriate use (misuse) of antibiotics: This constitutes one of the most important factors in drug resistance. Inappropriate antibiotics use could be as a downstream consequence of excessive drug promotion activities in vulnerable global populations who may be devoid of information channels to make informed decisions on benefits and risks of antimicrobials (Olivier *et al.*,2010).Inappropriate use of antibiotics is of three types.

(i) Wrong choice of prescription or use in person without discernible signs of infection: It is now estimated that 50% of antibiotics prescriptions are associated with inappropriate use, either because of unnecessary length of treatment, wrong choice of prescription or dosage regimen, or use in persons without a discernible sign of infection (Olivier *et al*., 2010)

(ii) Overuse of Antibiotics: Most antibiotics have become ineffective against their target organism because they are widely overused without a sound rational indication of their prescription. The frequent prescription of broad spectrum antibiotics in place of a better targeted antibiotic through more precise diagnosis has also contributed to overuse of drugs.

(iii) Underuse or erratic/inconsistent self administration. This can result from 'drug holidays' in patients who stop taking their medication after an initial favorable therapeutic response or acute side effects, only to return to their physicians' office with a recurring infection by a more resistant strain of bacteria. Such erratic use or underuse of antibacterial is likely to be more predominant in developing countries, where patients often lack both the financial resources and general knowledge about pharmaceutical use to adhere to their antibacterial treatment regimen (Olivier *et al.*, 2010).

Other social factors that are widespread in developing countries such as low quality generic or counterfeit antimicrobials, ineffective distribution and inappropriate storage conditions of pharmaceuticals can also contribute to erratic or suboptimal drug use and these factors can have even more drastic consequences when compounded by aggressive drug promotion practices (Olivier *et al.*, 2010)

2.1.2Classification of Drug Resistance

Drug resistance is classified into two which include natural resistant and acquired drug resistant

(i) Natural drug resistance:

Natural resistance means that the bacteria are 'intrinsically' resistant to the antibiotics. This could be as a result of the presence of gene responsible for the resistant, lack of transport system or target for the antibiotics, or as a result of increase efflux activity. For example, Streptomyces has some genes responsible for resistance to its own antibiotic.(Dzidic *et al* 2008).

(ii) Acquired resistance: This occurs in bacteria that are usually sensitive to antibiotics, but are liable to develop resistance. Acquired resistance is often caused by mutations in chromosomal genes, or by the acquisition of mobile genetic elements, such as plasmids or transposeons, which carry the antibiotic resistance genes (Dzidic *et al* 2008).

2.1.3 Mechanism of Drug Resistance**.**

Multidrug Resistance (MDR) can be caused by different mechanisms in different organisms. There are four major classes of mechanisms of bacterial resistance to antibacterial agents:

Drug inactivation mechanisms: Bacteria have developed several mechanisms of rendering antibiotics inactive such as the enzymatic hydrolysis of antibiotics, group transfer and the redox process (Kumar and Varela 2013). The classical example of this mechanism is the production of β- lactamases that hydrolyze the β-lactam ring of beta lactam ring containing antibiotics such as penicillin (Kumar and Varela 2013). The production of β-lactamase by the bacteria rendered the first antibiotic penicillin ineffective, and the enzyme is thought to have some important role in cell wall peptidoglycan assembly. The genes encoding β-lactamases (*bla*) are either on the chromosome like in the case of AmpC β-lactamase or on a plasmid. The first plasmid mediated β-lactamase isTEM-1 β-lactamase which was discovered on the plasmid in a strain of *Escherichia coli*, followed by a second plasmid mediated β-lactamase SHV-1 (sulphydril variable active site) (Kumar and Varela 2013). TEM and SHV enzymes, later evolved to hydrolyze a broad range of extended spectrum cephalosporin, and are collectively called extended spectrum β-lactamases (ESBLs). TEM and SHV were the major ESBLs until the discovery of third unique cefotaxime degrading enzyme CTX-M type in 1990 in *E. coli*, and now there are more than 40 types of CTX-M ESBLs (Bonnet 2004). ESBLs hydrolyze a wide range of cephalosporin including the oxyimino group of cephalosporin such as ceftriaxone, ceftazidime, cefotaxime and the monobactam drugs such as aztreonam. Currently, more than 200 ESBL types have been discovered worldwide, and these have evolved from TEM, SHV and CTX-M types by point mutations (Gniadkowski 2001). Though Enterobacteriaceae such as *E. coli* and *Klebsiella pneumoniae* are the predominant producers of ESBLs, many Gram negative bacteria are now known to produce them. Pathogenic bacteria that are causative agents of urinary tract infections and other infectious diseases are capable of producing multiple ESBLs and thus, offer a great therapeutic challenge leaving the physicians with very few treatment options. Like

β-lactamases, bacteria produce other antibiotic hydrolyzing enzymes such as the macrolide esterase and fosfomycin epoxidases. Macrolide esterase produced by many members of Enterobacteriacease inactivate erythromycin and oleandomycin by hydrolyzing the lactone ring (Kumar and Verela 2013)

Target modification: Bacteria have found ways of resisting antibiotics by altering or modifying their antibiotics molecular targets such as DNA gyrase (a target for Quinolone), RNA polymerase (a rifampin target), prokaryotic ribosome (a tetracycline target).Example of this mechanism is the alteration of the penicillin binding protein (PBP) which is the target of β-lactam antibiotics by *Staphylococcus aureus* the causative agent of serious infectious disease such as urinary tract infection and respiratory infection. The organism becomes resistant to these antibiotics by any one of the several mechanisms such as mutation in PBP or acquisition of new PBP with reduced affinity to penicillin, over expression of PBP, (Fisher *et al*., 2005). Another example of an altered target mechanism includes substitution of amino acids in the quinolone resistance determining region (QRDR) of DNA gyrase andtopoisomerase IV resulting in less efficient binding of quinoloneantibiotics. This mechanism has been responsible for widespread quinolone resistance among the Enterobacteriaceae. Methylation of drug binding targets on 16S rRNA by rRNA methyl transferases is responsible for amino glycosideresistance in several bacterial species (Jana and Deb 2006). On the other hand, mutations in genes (*rrs*) encoding ribosomal subunits altered ribosomal protein targets which resist aminoglycoside binding, a mechanism responsible for streptomycin resistance in *Mycobacterium tuberculosis*, the causative agent of tuberculosis and other infections. Methylation of the 23S rRNA component of 50S ribosomal subunit by adenine specific N-methyltransferases is a common mechanism of macrolide resistance in many Gram-positive and negative bacteria (Lambert 2005). Also,

mutations around the methylated sites have also been responsible for additional macrolide resistance (Xiong *et al.,* 2000). The vancomycin resistant enterococci (VRE) have evolved a unique mechanism of synthesizing peptidoglycan using alternate pathway thereby producing the peptidoglycan precursors ending with acyl-D-Ala4-D-Lac5 instead of the vancomycin target acyl D-Ala4-D-Ala5(Reynolds and Courvalin 2005).

Reduced permeability, Drug resistance bacteria may be due to the inability of the antimicrobial agent to gain entry into the cell where the drug targets are located (Kumar and Schweizer 2005). One mechanism that results in reduced drug permeability in bacteria is the presence of cell wall's lipopolysaccharide (LPS), which consists of lipid A, polysaccharide and O-antigen. Bacteria that harbor LPS moieties show resistance to erythromycin, roxithromycin, clarithromycin and azithromycin in Gram-negative bacteria such as strains of *Pseudomonas aeruginosa*, *V. cholera* and *S. enterica*.(Strateva and Yordanov 2009). Another mechanism that confers reduced permeability involves the porin channels that reside in the outer membrane and allow small molecular weight molecules, such as antimicrobial agents to gain cellular entry (Pages *et al.,* 2008). Drug resistant bacteria alter the expression of these outer membrane proteins such that they fail to integrate into the outer membrane or are functionally defective, thus preventing the entrance of growth inhibitory molecules. Clinically important bacterial pathogens like *Serratiamarcescens, E. cloacae, S. enterica, E. aerogenes, Klebsiella pneumoniae*, and *P. aeruginosa*, have utilized this reduced drug uptake system to resist important antimicrobial agents, such as the beta-lactams, fluoroquinolones, aminoglycosides, as well as chloramphenicol (Page 2012).

Active drug efflux; one of the most common drug resistance mechanisms is active efflux of drugs from inside of bacterial cells (Webber,and Piddock 2003). Such drug resistant bacteria

possess energy driven drug efflux pumps which extrude antimicrobial agents thus reducing their intracellular concentrations to non inhibitory levels. There are two main types of active efflux pumps.

1. Primary active transport. It uses the energy from hydrolysis of ATP to actively efflux drugs from cells,

2. Active transport: It uses an ion gradient for active drug efflux from cells (Davidson and Maloney 2007). The ATP driven transporters are also known as ABC (for ATP-binding cassette) or P-glycoprotein transporters. Both active transport systems are used by bacteria to resist the inhibitory effects of antimicrobial agents and are often referred to as efflux pumps (Kumar and Varela 2013). In addition to single drug efflux pump systems, bacteria may also express efflux pumps that are able to extrude multiple structurally different antimicrobial agents and are referred to as multidrug efflux pumps (Kerr *et al.*, 2010). These efflux pumps function by using the energy of the cation gradient generated by cellular respiration to catalyze the "uphill" transport of solute (e.g drug substrate) across the membrane by translocation of the cation (e.g H+ or Na+) down its concentration gradient in a process called antiport, where cation moves in one direction across themembrane and drug (substrate) moves in the opposite direction (Kumar and Varela 2013).

2.2 Antibacterial Agents

Antimicrobial agents are those natural or synthetic substances that inhibit microorganism growth kill or destroy microorganism. These organisms could be bacteria, fungi, or viruses. The commonly use antimicrobials agent are antibiotics. Antibiotics act selectively by interacting with the target present on the bacterial cell (prokaryotes) which is absent on the mammalian host. For the ß-lactam drugs, this target is the cell wall. Mammalian cells lack cell walls, which for the bacteria are a critically important barrier protecting the organism from osmotic stresses, heat and viruses and also as a protection from antimicrobial peptides, and host defense mechanisms. The major properties of an antibiotic that determine its activity against specific bacteria are: affinity of the drug for the target, the permeability of the drug, and stability to bacterial enzymes that inactivate or destroy the drug (Benton *et al*., 2007).

2.2.1Modes of Action of Antibiotics

There are five major modes of antibiotic mechanisms of action

Interference with cell wall synthesis: ß-lactam antibiotics such as penicillin and cephalosporin interfere with enzymes required for the synthesis of the peptidoglycan layer. Glycopeptides target the bacterial cell wall by binding to the D-alanyl-D-alanine termini of the peptidoglycan chain, thereby preventing the cross-linking steps (Benton *et al*., 2007).

Inhibition of protein synthesis: Some antibiotics act on bacteria by inhibiting the protein synthesis at different stages. A wide range of antibiotics interfere with protein synthesis on different levels of protein metabolism (Dzidic *et al.,* 2008). For example Macrolides bind to the 50S ribosomal subunit and interfere with the elongation of nascent polypeptide chains. Amino

glycosides inhibit initiation of protein synthesis by binding to the 30S ribosomal subunit. Chloramphenicol binds to the 50S ribosomal subunit blocking peptidyl transferase reaction. Tetracycline inhibits protein synthesis by binding to 30S subunit of ribosome, thereby weakening the ribosome tRNA interaction (Dzidic *et al* 2008).

Interference with nucleic acid synthesis: Antibiotics also inhibit bacteria growth or kill the bacteria by interfering with the nucleic acid synthesis of the bacteria. For example Rifampicin interferes with a DNA-directed RNA polymerase. Quinolones disrupt DNA synthesis by interfering with type II topoisomerases DNA gyrase and topoisomerase IV during replication and by causing double strand breaks (Benton *et al* 2007).

Inhibition of a metabolic pathway: The sulfonamides (*e.g.* sulfamethoxazole) and trimethoprim each block the key steps in folate synthesis which is a cofactor in the biosynthesis of nucleotides the building blocks of DNA and RNA (Dzidic *et al* 2008).

Disorganizing of the cell membrane The primary site of action of antimicrobial agent is the cytoplasm membrane of Gram positive bacteria or the inner membrane of Gram negative bacteria. For example polymyxins exert their inhibitory effects by increasing bacterial membrane permeability causing leakage of bacterial content. The cyclic lipopeptide, daptomycin displays rapidbactericidal activity by binding to the cytoplasmic membranein a calcium dependent manner and oligomerizingin the membrane, leading to an efflux of potassiumfrom the bacterial cell and cell death (Tenover 2006).

2.3 Drug Discovery from Natural Source

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources. Many of these isolations were based on the uses of the plants in traditional medicine(Vashist and Jindal 2012).Plants, animals and microorganisms are reservoir of natural products, or natural sources for compounds used as remedies for several diseases in many parts of the world (Brusotti *et al.,* 2013). About 60% of the world's population depends on traditional medicines as the primary health care system (Vashist and Jindal 2012). Plant species with possible biological activity remain a large source Newman and Cragg (2012) reported that natural product continued to play a highly significant role in the drug discovery and development process. Thus, biodiversity represents an unlimited source of novel chemicals entities (NCE) with potential as drug leads. These NCE are secondary metabolites synthesized by plants as defense against herbivores and pathogens or attraction of pollinating agent, and can be grouped in three main chemical families: alkaloids, terpenoids and phenolic compounds. A review from (Kashani *et al.,* 2012) recently highlighted the pharmacological properties of some well known secondary metabolites such as alkaloids, terpenoids and phenolic compounds giving a direct evidence of the crucial role of natural products as potential sources of various modern pharmaceuticals. However, secondary metabolites are often present in low quantity in plant material and their extraction, purification and characterization still remain a great step in the drug discovery process.

2.3.1 The use of plants in ethno medicine for drug discovery:

The use of plants for diseases treatment is as old as the human species. Observations on the use and efficacy of medicinal plants have significantly contributed to the disclosure of their therapeutic properties, so that they are frequently prescribed traditionally even if their chemical constituents are not always completely known. The knowledge of their healing properties has been transmitted over the centuries within and among human communities. Active compounds produced during secondary vegetal metabolism are usually responsible for the biological properties of some plant species used throughout the globe for various purposes, including treatment of infectious diseases (Silva and Fernandes 2010).

According to the World Health Organization (WHO, 1977) "a medicinal plant" is any plant which one or more of its organ contains substances that can be used for the therapeutic purposes or which are precursors for the synthesis of useful drugs. Medicinal plants are plants containing inherent active ingredients used to cure disease or relieve pain (Okigbo *et al*., 2008). Most developing countries observe the use of traditional medicines and medicinal plants as therapeutic agents for the maintenance of good health. Modern pharmaceuticals still contains at least 25% drugs derived from plants and many others which are synthetic analogues built on prototype compounds isolated from plants. The growing interest in medicinal plants for maintenance of good health and well being could be as a result of the rising costs of prescription drugs, escalating faith in herbal medicine, access to conventional drugs, and side effects of the synthetic drugs (Okigbo *et al*., 2008). According to World Health Organization, medicinal plants would be the best source to obtain a variety of drugs. Therefore, such plants should be investigated to better understanding of their properties, safety and efficacy (Nascimento *et al*., 2000). Medicinal plants produce bioactive compounds used mainly for medicinal purposes. These compounds either act on different systems of animals including man, or act through interfering in the

metabolism of microbes infecting them. The microbes may be pathogenic or symbiotic. In either way the bioactive compounds from medicinal plants play a determining role in regulating host microbe interaction in favor of the host. So the identification of bioactive compound in plants, their isolation, purification and characterization of active ingredients in crude extracts by various analytical methods is important. The medicinal properties of plants could be based on the antioxidant, antimicrobial, antipyretic effects of the photochemical in them (Adesokan *et al*., 2008).

2.4The Plant *Zizyphus lotus*

(fig 2.1)

Zizyphus lotus belongs to genus Zizyphus of about 40 species. Among the species are: *Ziziphus murtiana*, *Ziziphus sativa, Ziziphus spinachristi, Ziziphus nummularia, Ziziphu sjujube*, *Ziziphus oxyphylla*. It belongs to the family Rhamnaceae.It is distributed in the warm-temperate and subtropical regions throughout the world (Abalaka *et al.,* 2010). It is a spiny shrub and small tree with buck thorns. It grows about 5-7 m height, the leaves are alternate, entire with three prominent basal veins. It is found in many habitats in arid and semi arid regions. It grows vigorously in drought condition because of its rapidly developing taproot. Plants are erect or wide spreading with drooping thorny branches, short sharp straight or hooked spines. It is dark green and glossy on the upper side and pubescent and pale green to grey green on the lower side. The flowers are small, yellow, and five petals which are usually in twos and threes in the leaf axils. This fast growing tree starts producing fruits within three years. Plant flowers late in spring from October to December and fruitifies from November to March. The fruits are an edible

drupe. The endocarp is edible sweet mucilage. The fruit of *Ziziphus lotus* is a globosely dark yellow drupe 1–1.5 cm diameter (Abdeddaim *et al.*,2014).



Fig. 2.1 *Ziziphus lotus* Plant

2.4.1 Medicinal Properties of *Ziziphus lotus*:

Several parts of *Ziziphus lotus* have been used traditionally in many part of the world for treatment of several pathologies such as digestive disorders, weakness, liver problems, obesity, urinary infections, diabetes, skin infections, fever, diarrhea and insomnia (Benammar *et al*., 2014). *Ziziphus lotus* is also used in traditional medicine for its antidiabetic, sedative and hypoglycemic activities. The medicinal properties of this plant depend on the part of the plant concerned (root, leaf stalk and pulp or fruit) and the extract used. Fruit has been used for its emollient properties, and a mixture of dried leaves and fruits is applied in the treatment of boils (Benammar *et al*., 2014).The root bark is known for its antidiabetic activity. Bark of root of Ziziphus *lotus* given intraperineally showed a significant and dose dependent anti inflammatory and analgesic activity in carrageenan induced paw edema in rat. (Borg*i et al*., 2007). (Bakhtaoui *et al* 2014) reported that methanol extract of *Ziziphus lotus* fruit showed significant positive effect on gastric ulcer induced in rats. The leaf and root extracts corrected antioxidant status of diabetic animals in pancreas, liver and erythrocytes (Benammar *et al.,* 2014).

2.4.2 Nutritional properties of *Ziziphus lotus*

The fruit of *Zizyphus lotus*is made up of two parts the pulp and almond. These parts of the fruits have been studied for their nutritional content and the following result was obtained. The pulp (P)has3.80%Crude protein,1.32%crude fat, 8.41% crude fiber, 3.28% ash, 65.90%carbohydrate, 3.78% pectin, 12.27% moisture contents and 16.341 Kj/g Caloric value. While the almond (A) has 24.22%, Crude Protein, 31.73% Crude fat, 16.57% Crude fibre, 3.12% ash, 11.10% carbohydrate, 2.35% pectin, 9.57% moisture content and 17.271Kj/g caloric value(Abdeddaim*et al*.,2014).The presence of glucose, fructose and sucrose were detected in the pulp. The amino

acids profile of the fruit revealed the presence of cysteine, glycine, arginine, serine, leucine, histidine and alanine. The almond is richer in fats and the fatty acids analysis revealed the presence of 15 fatty acids; the unsaturated ones were at 71% with 49% of oleic acid of the total mixture, followed by 22% of linoleic acid (Abdeddaim *et al*., 2014). The elemental analysis of the two parts of the fruit in mg/100g dry matter (DM) indicated that the (P) contained appreciable levels of zinc (0.44), potassium (134.99), sodium (11.45), phosphorus (10.62), manganese (2.17), magnesium (397.91) and iron (1.33). The (A) appears to be richer in these elements, especially phosphorus (24.00) and magnesium (1349.06). These results revealed that this fruit presents a high nutritional value (Abdeddaim *et al*.,2014).

2.5 Phytochemicals found in *Ziziphus lotus*

Phytochemicals are chemical compounds formed during the plants normal metabolic processes.

Phytochemicals accumulate in different parts of the plants, such as the roots, stems, leaves, flowers, fruits or seeds (Saxena *et al.*, 2013). Many phytochemicals particularly the pigment molecules are often concentrated in the outer layers of the various plant tissues. Levels vary from plant to plant depending upon the variety. These chemicals are also called "secondary metabolites" and have biological properties such as antioxidant activity, antimicrobial effect, modulation of detoxification enzymes, stimulation of the immune system, decrease of platelet aggregation and modulation of hormone metabolism and anticancer property (Saxena *et al.*,2013). Phytochemicals are classified as primary or secondary constituents depending on their role in plant metabolism. Primary constituents include the common sugars, amino acids, proteins, purines and pyrimidines of nucleic acids, chlorophyll's etc. Secondary constituents are the alkaloids, terpenes, flavonoids, lignans, plant steroids, curcumines, saponins, phenolics,

tanninsand glucosides. (Saxena *et al.,* 2013). Phytochemical screening of crude extracts of leaf and root of Ziziphus lotus revealed the presence of Alkaloids, flavonoids, Saponins, Tannins, Cardiac glycosides and cyanogenicglycosides

2.5.1 Flavonoids:

Flavonoids are polyphenolic compounds that are ubiquitous in nature. Many of which occur in vegetables, fruits and beverages like tea, coffee and fruit drinks (saxena *et al*., 2013). Flavonoids contribute to the medicinal properties of plants and appear to have played a major role in successful medical treatments of ancient times, and their use has persisted up to now(saxena *et al*., 2013). Flavonoids have broad biological and pharmacological activities such as antimicrobial, anti-inflammatory as well as antitumor activities but the best described property of almost every group of flavonoids is their capacity to act as powerful antioxidants which can protect the human body from free radicals and reactive oxygen species (saxena *et al*., 2013). The capacity of flavonoids to act as antioxidants depends upon their molecular structure. The position of hydroxyl groups and other features in the chemical structure of flavonoids are important for their antioxidant and free radical scavenging activities (Tapas *et al.,* 2008).

2.5.2 Tannins:

Tannins are a heterogeneous group of high molecular weight polyphenolic compounds with the capacity to form reversible and irreversible complexes with proteins, polysaccharides (cellulose, hemicelluloses, pectin,), alkaloids, nucleic acids and minerals(Schofield *et al.*, 2001). Tannin can be divided in to four major groups based on their structures: Gallo tannins, ellagitannins, complex tannins, and condensed tannins (Saxena *et al*., 2013).

(1) Gallotannins are all those tannins in which galloyl units or their metadepsidic derivatives are bound to diverse polyol, catechin, or triterpenoid units.

(2) Ellagitannins are those tannins in which at least two galloyl units are C–C coupled to each other and are glycosidically linked.

(3) Complex tannins are tannins in which a catechin unit is bound glycosidically to a Gallotannin or an ellagitannin unit.

(4) Condensed tannins are all oligomeric and polymeric proanthocyanidins formed by linkage of *C*-4 of one catechin with *C*-8 or *C*-6 of the next monomeric catechin. Tannins are found commonly in fruits such as grapes, persimmon, blueberry, tea, chocolate, legume forages, legume trees like *Acacia* spp., *Sesbania*spp, sorghum, corn, etc(Serrano *et al.*, 2009). Several health benefits have been recognized for the intake of tannins and some epidemiological associations with the decreased frequency of chronic diseases have been established. Tannin containing plant extracts are used as astringents, against diarrhea, as diuretics, against stomach and duodenal tumours and as anti-inflammatory, antiseptic, antioxidant and haemostatic Pharmaceuticals (Dolara *et al.*, 2005). Tannins are used in the dyestuff industry as caustics for cationic dyes (tannin dyes), and also in the production of inks (iron gallate ink). In the food industry tannins are used to clarify wine, beer, and fruit juices. Other industrial uses of tannins include textile dyes, as antioxidants in the fruit juice, beer, and wine industries, and as coagulants in rubber Production. Recently the tannins have attracted scientific interest, especially due to the increased incidence of deadly illnesses such as AIDS and various cancers (Saxena *et al*., 2013).

2.5.3 Alkaloids:

Alkaloids are natural product that contains heterocyclic nitrogen atoms. They are basic in nature. Alkaloids protect plants against micro-organisms (antibacterial and antifungal activities), insects and herbivores (feeding deterrens) and also against other plants by means of allelopathically active chemicals (Saxena *et al.* , 2013). Alkaloids containing plants are used as dyes, spices, or drugs. Alkaloids have many pharmacological activities such as antihypertensive effects, antiarrhythmic effect (quinidine, spareien), antimalarial activity (quinine), and anticancer actions. Some alkaloids have stimulant property such as caffeine and nicotine, morphine are used as the analgesic and quinine as the antimalarial drug (saxena *et al*., 2012).

2.5.4 Saponins:

Saponins are a group of secondary metabolites found widely distributed in the plant kingdom. They form a stable foam in aqueous solutions such as soap, hence the name saponin.The physiological role of saponins in plants is not yet fully understood (Saxena *et al.*, 2013). Many saponins are known to be antimicrobial, to inhibit mould, and to protect plants from insect attack. Saponins may be considered a part of plants' defense systems, and as such have been included in a large group of protective molecules found in plants named phytoanticipins or phytoprotectants (Lacaille-Dubois and Wagner 2000).Saponin mixtures present in plants and plant products possess diverse biological effects when present in the animal body. Extensive research has been carried out into the membrane permeabilising, immune stimulant, hypocholesterolaemic and anticarcinogenic properties of saponins and has also been found to significantly affect growth, feed intake and reproduction in animals. These structurally diverse compounds have also been observed to kill protozoan and molluscs, to be antioxidants, to impair the digestion of protein and

the uptake of vitamins and minerals in the gut, to cause hypoglycemia, and to act as antifungal and antiviral (George *et al.,* 2002).

2.6 Overview of The test Bacteria

2.6.1 *Escherichia coli*

This is a Gram-negative, facultative anaerobic, rod-shaped bacterium that is commonly found in the lower intestine of warm blooded organisms (endotherms). Most *E.coli* strains are harmless, but some serotypes such as enterotoxigenic E. coli, enteropathogenic *E.coli*, enteroinversive *E. coli,* and enteroaggregative *E. coli* can cause disease to humans. The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K, and by preventing the establishment of pathogenic bacteria within the intestine (Zinnah *et al.,* 2007).

Scientific classification

Domain: Bacteria

Kingdom: Eubacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Enterobacteriales

Family: Enterobacteriaceae

Genus: *Escherichia*

The commonest infection caused by *E coli* is urinary tract infection and Diarrheal infections. The organism normally spread from the gut to the urinary tract. It also cause cystitis (infection of the bladder), and in some cases the infection may spread up the urinary tract to the kidneys causing pyelonephritis (Zinnah *et al.*,2007).Patients in hospital who have catheters or tubes placed in the urethra and bladder are also at risk of infection by *E. coli*. Overspill from the primary infection sites to the bloodstream may cause blood poisoning (*E. coli* bacteraemia). In rare instances, *E. coli* may cause meningitis in very young children.(Turner *et al.,* 2006**).**

Escherichia coli can also develop resistance to antibiotics through mechanism such as enzymatic Inactivation of antibiotics. *E. coli* produced ß Lactamase an enzyme that hydrolyses ß Lactam ring of the antibiotics. TEM-1 ß-lactamase was the first ß-lactamase to be discovered on a plasmid in a strain of *E. coli* followed by SHV -1(sulphydril variable active site) as the second type of ß-lactamase also on a plasmid in a strain of *E. coli*. TEM and SHV later evolved to hydrolyse broad spectrum antibiotics and are collectively called extended spectrum ß-lactamases (ESBLs) (Kumar and Varela 2013).

2.6.2*Pseudomonas aeruginosa*

It belongs to the family Pseudomonadaceae, genus *Pseudomonas* and species *Pseudomonas aeruginosa.* It is a gram negative aerobic organism. Rod shape measured 0.5 to 0.8µm. It can be identified *invitro* by its specific odor and blue greenish colour producing colonies. The optimum growth temperature of this organism is 37^0C and the maximum is 42^0C. The strains move by means of polar flagellum. The organism is wide spread in natural habitat and can be isolated from different sources like water, soil, plants, animals and humans (Goldberg and pier 2000).

The organism is clinically important as it is opportunistic pathogen that causes disease in animals and human (Chugani and Greenberg 2007). It can cause different infections both acute and chronic. It can invade any part of the body. It commonly infects respiratory tract of immune compromised patient and people with cytic fibrosis disease *P. aeruginosa* produces enzymes and toxins that break down barrier to enter and damage host cells, resist phagocytosis and host immune defenses. In addition, the polysaccharide slime and sort of false capsule produced by P. *aeruginosa* effectively protects cells from opsonization by antibodies, complement deposition, and phagocyte engulfment (Behar *et al.,* 2008). Elastase and alkaline protease destroy the ground substance of the cornea and other supporting structures composed of fibrin and elastin resulting in invasion and injury. *Pseudomonas aeruginosa* produces three other soluble proteins involved in invasion: a cytotoxin and two hemolysins (Ciofu *et al.,* 2001).The cytotoxin is a pore-forming protein that has effect on neutrophils, but it appears to be cytotoxic for most eucaryotic cells. The two hemolysins, one is a phospholipase and the other is a lecithinase. They appear to act synergistically to break down lipids and lecithin. The cytotoxin and hemolysins contribute toinvasion through their cytotoxic effects on neutrophils, lymphocytes and othereucaryotic cells. Pyocyanin the pigmen impairs the normal function of human nasal cilia and disrupts the respiratory epithelium (Ciofu *et al.,* 2001). *P. aeruginosa* confer resistance to antibiotics by producing broad spectrum ß-lactamases, Metallo ß-lactamases, (MBLs), alteration of protein binding penicillin (PBP), porin mutation, formation of biofilm, and efflux pump (Ochoa *et a*l., 2013).

2.6.3 *Salmonella* species

Salmonella species is a facultative anaerobe, gram negative flagellated rod-shaped bacterium which is about 2-3 x 0.4-0.6μm in size (Pui *et al.,* 2011). It is non-capsulated. It is among the most commonly isolated food borne pathogens associated with fresh fruits and vegetables. Salmonellae are non-fastidious as they can multiply under various environmental conditions outside the living hosts. Most *Salmonella* serotypes grow at temperature range of 5 to 47°C. It belongs to the genus salmonella, family enterobacteriaceae. The bacteria infects only human. The main rout of transmission is through water, fruits, vegetables, and food (Pui *et al.,* 2011). *Salmonella* species causes typhoid fever while paratyphi A,B, and C caused paratyphoid fever both serotypes are solely human pathogens. Infection occurs as a result of ingestion of food or water contaminated with the bacteria since *Salmonella typhi* and *paratyphi* do not have animal reservoir (Newell *et al*.,2010).The patient may relapse die or encounter serious complication such as typhoid encephalopathy, gastrointestinal bleeding, and intestinal perforation (Hu and kopeko, 2003: Parry, 2006).Salmonella species has the following antigenic features.

Somatic antigen (O): This represents the side chain of repeating sugar units projecting from the outer lipopolysaccharide layer of the bacteria cell wall.

Flageller antigen (H): which are the determinant groups in the flageller proteins They are heat labile as well as alcohol labile but heating at 60^0C and above cause detachment of the flageller from the bacteria.

3 Vi- antigen: Almost all strain of salmonella typhi produce the Vi- antigen as a covering layer outside their cell wall. It is an acidic polysaccharide and when fully developed it renders the bacteria agglutinable to Vi-antibody (Pui *et al.,* 2011).Resistant of *Salmonella* to single antibiotic

was first reported in 1960s (Pui *et al.,* 2011). Since then more resistant straint of Salmonella has been isolated in different parts of world. Resistant to first-line antibiotics such as ampicillin, chloramphenicol and trimethoprim- sulfamethoxazole defined multidrug resistant in *Salmonella* species (Pui *et al.*, 2011).

2.6.4 *Staphylococcus aureus*

Staphylococcus aureus are both commensal and pathogenic. It is gram positive bacteria. Coccus shaped microorganism with diameter of between 0.7 m to1.2m that generally occurs in grape-like clusters, but can also be found in singles and pairs. They are non-motile, non-spore forming, facultative, anaerobic organism (Bachrrho and Abouni 2015).They are catalase and coagulase positive but oxidase negative.*Staphylococcus aureus* grows best under aerobic conditions but is able to employ a fermentative metabolism, making it a facultative anaerobe (Ryan 2004).

The organism is able to utilize several different carbohydrates during respiration. However, under anaerobic conditions, *S. aureus* will typically ferment glucose resulting in the production of lactic acid and has ability to ferment mannitol. Members of the species grow in the presence of salt, under high osmotic pressure, low moisture and at wide pH and temperature ranges (Bachrrho and Abouni 2015).A unique characteristic of *S. aureus* is its ability to grow in saltconcentrations as high as 15%. The optimum water activity for *S. aureus* is about 0.99 but it is also capable of growing at water activities as low as 0.83 (Bachrrho and Abouni 2015).if other intrinsic and extrinsic factors are optimal for growth of this pathogen. *Staphylococcus aureus* is capable of growing in temperatures ranging from 7 to 48.5°C with an optimum growth temperature of 30 to 37°C (Bachrrho and Abouni 2015). In addition to being able to grow in wide range of temperatures, *S.aureus* can also grow in a wide range of pH values ranging from

4.2 to 9.3 with an optimum pH of 7 to 7.5. As with all Gram positive bacteria, the cell wall contains peptidoglycan. However, it also contains ribitoltechoic acid molecules that are specific to *S. aureus* and act as antigens (Ryan 2004). In addition to the presence of ribitol techoic acid in the cell wall of *S. aureus*, some strains may also possess the protein A, that can comprise up to 7 % of the cell wall and may coat the outside of the cell. Both ribitol techoic acid and protein A work to increase the virulence of the microorganism (Ryan 2004). The cell wall of *S. aureus* is also very thick in comparison with other gram positive bacteria. This increased thickness provides the organism with a very high internal pressure making it nearly impossible for many antimicrobial drugs to enter the cell (Bachrrho and Abouni 2015). *S. aureus* can easily distinguish from pathogenic streptococci by its production of catalase. However, it is relatively similar to the other less virulent staphylococci. Due to this similarity *S. aureus* must be distinguished via diagnostic tests. Most strains of *S. aureus* will exhibit β- hemolysis when grown on blood agar which can be a distinguishing characteristic. *S. aureus* is differentiated from other staphylococcal species on the basis of coagulase reaction (coagulase positive). Coagulase binds to prothrombin in the blood causing fibrin to be polymerized, resulting formation of a clot. (Ryan 2004). The coagulase produced by *S.aureus*is considered a determinant of the pathogenicity of the organism .They form part of the normal bacterial flora of the skin and mucosal surface of the respiratory tract, upper alimentary tract of mammals (Bachrrho and Abouni 2015). Staphylococcus aureus resistant penicillin was first reported in 1942 in hospital sample and subsequently in community (Bachrrho and Abouni 2015). Methicillin a penicillinase resistant semi synthetic penicillin was introduced in 1961 but the resistant strain of *S. aureus* to this drug emerged immediately known as methiccillin resistant *S. aureus* (MRSA) (Bachrrho and

Abouni 2015). S. aureus confer resistance to other antibiotics like Cephalosporin and Quinolone by producing ß lactamase (Bachrrho and Abouni 2015).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Chemicals/ Reagents.

Methanol, n-hexane, Ethyl acetate, n-butanol (JHD, AR: Lobal. Chem, India) were obtained from Haddis Chemical Laboratory. Thin layer chromatographic plate (TLC) silica gel 60 pre coated plate, Silica gel for column chromatography (60-120μm in size) were also obtained from the same Laboratory, Sulphuric acid and Para anisaldehyde. Mueller Hinton Agar, Mueller Hinton nutrient agar and Nutrient broth were also obtained from Chemical lab. All chemicals were obtained from haddis chemical Laboratory India and are of analytical grade

3.2 Equipments

Water bath (HHS,Mc Donald Scientific international), Autoclave, Flasher Scientific Incubator and Q10 GC-MS SHIMAZDU

3.3 Collection and Preparation of Plant extracts

3.3.1 Collection of plant materials:

Leaf and root of *Ziziphus lotus* were collected from Quaanpan local government area Plateau state and taken to Herbarium unit Botany Department Ahmadu Bello University Zaria for identification Voucher number of V/No1283 was given. The leaf and root of the plant obtained was washed with tap water and air dry under room temperature. After drying, it was grounded in to powder using mortar and pestle.

3.3.2 Extraction of plant materials

The extraction of plant materials was done using cold maceration method. One kilogram (1kg) of powdered leaf and root were soaked in 1.5 L of methanol separately and kept under room temperature with manual shaking after every two hours during the day for 48 hours (Diallo *et al*., 2008). The mixture was then filtered using whatman No 1 filter paper and filtrate was concentrated under vacuum with rotary evaporator to 40 ^{0}C. The extract was then kept for further use.

3.3.3 Preparation of different concentration of plant extracts

The different concentrations (200, 100, 50, and 25 mg/ ml) of the extracts used during the study were prepared by dissolving 2 g of each extract in 10 ml of 5% Dimethyl sulphoxide(DMSO) to get 200 mg/ml concentration. Other concentrations 100, 50, and 25 mg/ml were prepared using two fold dilutions using 5% DMS as the solvent. Ciprofloxacin of concentration 10µg was used as standard drug and 5% DMSO was used as the Negative control.

3.4 Collection and Identification of Test Bacteria

The following bacterial isolate: *Salmonella* species, *Escherichai coli, Staphylococcus aureus*, and *Pseudomonas aeruginosa* were collected from Ahmadu Bello University teaching hospital Zaria and taken to Microbiology Department Laboratory for further authentication. The Isolates were subcultured and subjected to antibiotics susceptibility test using the following antibiotics: Erythromycin, Gentamycin, Amoxicillin, Tetracycline, and Ciprofloxacin to confirm their resistance to the antibiotics.

3.5 Phytochemical Determination of Crude Extracts

3.5.1 Qualitative determination of phytochemical content of extract

I Test for Saponins (frothing test)

Exactly 0.5 grams of each crude extracts was dissolved in distilled water. 2 milliliter of the prepared solution of each extracts was taken into a cleaned test tube and was vigorously shaken and left to stand for 10 min, the development of foam on the surface of the mixture lasted for more than 10 min indicates the presence of saponins

ii Test for Tannins (ferric chloride test).

Few drops of ferric chloride was added to one milliliter of the extracts the formation of dark green colour indicated a positive test for tannins (Santhi and Sengottuvel 2016).

Iii Test for Alkaloid (Dragendoff's test)

Few drops of Dragendoff's reagent were added to 1ml of the extract. The formation of orange red precipitate was taken as indicator of the presence of Alkaloids (Trease and Evans 1989).

Iv Test for Flavonoids (Sodium test)

One milliliter of the plant extracts was mixed with 2 ml of dilute Sodium hydroxid ethe formation of a golden yellow colour indicated the presence of flavonoids (Edeoga *et al.*,2005)

V Test for Cardiac Glycoside

Two milliliter of glacial acetic acid with few drops of iron (iii) chloride was mixed with 5 milliliter of the extract and 1 ml of concentrated sulphuric acid was added to the mixture. The

formation of brown ring with violet ring below it and green ring in the acetic acid layer indicated the presence of cardiac glycosides

3.5.2 Quantitative determination of phytochemical of crude extract

The following phytochemical: tannin, alkaloids, flavonoids, and saponins were determined from the extract using the method described by (Krishnaiah *et al., 2009.)*

I Determination of tannins

Exactly 50uL of tannin extracts for each sample was taken in a test tube and the volume was made up to 1ml with distilled water. Then 0.5 ml of Folin Clocalteu reagent was added and mixed properly and 2.5ml of 20% sodium carbonate solution was added, mixed and kept for 40 minutes at room temperature. Optical density was then taken at 725nm on the spectrophotometer and the concentrations was obtained from the standard curve. Percentage concenteration of tannin was determined using the expression:

$$percentage\ of\ Tannin = \frac{An \times C \times Df}{As \times W} \times 100$$

Where,

An = Absorbance of test sample

As = Absorbance of standard tannic acid

C = Concenteration of standard tannic acid (mg/ml)

Df = Dilution factor V_{ex}/V_a

W = Weight of test sample (mg)

V_{ex} = Volume of extract

V_a = Volume of extract analysed

ii Determination of alkaloids

Quantity of alkaloids was estimated using (Harbon 1973) method

Exactly 5 g of each crude extracts was placed in a 250 ml beaker and 200 ml of 10% ethanoic acid in ethanol was added. The mixture was covered and allowed to stand for 4 hours. It was then filtered and the filtrate was concentrated on a waterbath until it reaches a quarter of its original volume. Concentrated ammonium hydroxide was added until precipitation was complete. The mixture was allowed to settle and the precipitate collected on a weighed filter paper and washed with dilute ammonium hydroxide. The precipitate wasthen dried on the filter paper in an oven at 69^{o}C for 30mins and reweighed. The weight of alkaloid was determined using equation or expression below (Santhi and Sengottuvel 2016).

$$\%\ alkaloids\ = \frac{W2 - W1\ \times 100}{W}$$

Where, W_1=weight of empty filter paper

W_2=weight of precipitate and filter paper after drying

W=weight of extract.

Iii Determination of flavonoids

Ten grams of crude extract of leaf and root was repeatedly re- extracted with 100ml of 80% aqueous methanol at room temperature. The mixture was then filtered through a filter paper into a pre-weighed 250ml beaker. The filtrate was transferred onto waterbath and allowed to evaporate to dryness and weighed. The percentage flavonoid was calculated (Krishnaiah *et al.,* 2009)

iv Determination of saponins

Twenty grams (20g) ofleaf and rootcrude extract was weighed into a 250 ml conical flask and 100 milliliter of 20% ethanol was added. The mixture was heated over a hot water bath for 4 hours with continuous stirring at 55°C. It was then filtered through Whatman No.42 paper. The residue was re-extracted with another 200 ml of 20% C_2H_5OH. The combined extract was reduced to 40 ml over a waterbath set at 90 °C. The concentrated extract was then transferred into a 250 ml separation funnel and 20 ml of Diethyl ether $(CH_3CH_2)_2O$ was added to the extract and shaken vigorously. The aqueous layer was recovered while the diethyl ether $(CH_3CH_2)_2O$ layer was discarded. This purification process was repeated then 60 ml of n-butanol was added and the combined n-butanol extract was washed twice with 10 ml of 5% NaCl. The remaining solution was then heated on a waterbath in a preweighed 250 ml beaker. After evaporation the residue was dried in anoven to a constant weight. The saponin content was calculated as percentage of the weighed crude extracts (Salman *et al*., 2001).

3.6 Fractionation of crude extracts

Fractionation of crude extract was carried out using protocol design by (Kupchan and Tsou(1973) and modified by (Wagenen *et al.*,1993).

Exactly 80 grams of each crude extract was suspended in warm water to dissolve completely. The prepared solution was then partitioned successively using the following solvents of increasing polarity; n-hexane, ethyl acetate and n-butanol. Then 200 ml of n- hexane was added to each of the solution in a separation funnel and shacked for two layers of the mixture to form. The n- hexane portion was separated, and the process was repeated three times with the aqueous portion until a clear portion of the n- hexane was obtained. The same process was carried out using ethyl acetate and n- buthanol respectively to obtain the following fractions, n- hexane fraction, ethyl acetate fraction, n- buthanol fraction, and aqueous fraction. All fractions were evaporated to dryness using vacuum rotary evaporator and kept for further use (Ejele *et al.*, 2012).

3.7 Column chromatographic purification of active fraction

The chromatographic column with diameter 4 cm was washed with small amount of ethyl acetate and 100 g of silica gel G (60-120µm mesh size) was weighed and dissolved in ethyl acetate to form slurry. The column was carefully packed by adding small volume of ethyl acetate followed by the prepared slurry and allowed for some time to settle sufficiently as the stationary phase. Then 4 grams of plant extra was weighed and mixed with small quantity of silica gel to lose the sample for easy penetration of the solvent and loaded to the packed column and allowed to stabilize for some time before elution started. Gradient elution started with 500 ml 100% ethyl acetate, fractions were collected 50 ml per fraction. Subsequently the polarity was increased

using methanol in the following ratios ethyl acetate: methanol (2:1), (1:1), (1:2), (0:1).The fractions were numbered according to how they were collected and allowed to evaporate at room temperature. Fifty (50) fractions were collected from each extract. The column fractions were monitored on thin layer chromatography and visualized under U.V light. Para-anisaldehyde and 10% sulphuric acid was used as the general detecting reagent. Fractions that showed the same spot, cooler, and R_f value were combined together to form one fraction (Ejele *et al.,* 2012). After the combination of the TLC fraction both the root and leaf fraction formed four fractions each and were labeled $A_{R,}$ B_R, C_R and D_R for root and A_L, B_L, C_L and D_L for leaf respectively.

3.8 Antibacterial Susceptibility Test

3.8.1 Standardization of Bacteria Isolate**.**

Test bacteria were sub cultured and incubated at 37 ^{0}C for 24 hours. The discrete colonies from the sub cultured isolate were collected using sterile wire loop and introduced into 5 ml of normal saline and standardized by comparing the turbidity of the bacterial suspension with

0.5 Mcfarland standards to obtain 1.5 X 10^8cfu/ml bacteria culture.

3.8.2 Antibacterial susceptibility Test of crude extract and fraction

Susceptibility test of the organism was carried out using Agar well diffusion method

(Rojas *et al.,* 2006). Exactly 0.2 ml of standardized inocula was dispensed into each prepared petri dish with media using syringe and was spread using sterile swab stick. Four wells were made in each plate using sterile cork borer 6 mm diameter. Exactly 0.2 ml of the prepared extract was dispensed into each well according to the decreasing concentrations. The plates were

allowed to stand for one hour to allow adequate diffusion of the extracts, and then incubated at 37 ^{0}C for 24 hours. Each test was done in triplicate. Antibacterial activity was determined by measuring the zones of growth inhibition (clear zone) surrounding the wells in millimeter using transparent meter rule. 5% Dimethyl sulphoxide (DMSO) was used as the negative control. Ciprofloxacine 10 microgram concentration was used as standard drug.

3.8.3 Determination of minimum inhibitory concentration (MIC)

The Minimum Inhibitory Concentration was determined using broth dilution technique. Varying concentrations of the extracts (50, 25, 12.5, 6.25, and 3.13 mg/ml) were prepared and 1 ml of each concentration was introduced into 9 ml of nutrient broth in test tubes. Exactly 0.1 ml of the 24 hrs cultured standardized bacteria containing 1.5×10^8 CFU/ml was added and incubated at 37 ^{0}C for 24 hours. The least concentration of the extract that did not permit growth in the broth was taken as the minimum inhibitory concentration (Adeniyi *et al.*,1996).

3.8.4 Determination of minimum bactericidal concentration (MBC)

Minimum bactericidal concentration was determined using spread plate method. A fresh solid medium was inoculated with inocula from the concentrations that showed no visible growth during MIC. Exactly 0.2 ml from each test tube that showed no visible growth in MIC was dispensed into each plate and spread with sterile swab stick and incubated for 24 h at 37ºC. The lowest concentration in which no growth occurs on the solid medium was considered the minimum bactericidal concentration (Adesokan *et al*., 2007).

3.9Analysis of Data

The data obtained was analyzed using SPSS windows version 20.The analysis was performed using one way ANOVA, Duncan multiple range tests was used to compare significant difference within the groups with P- value < 0.05 considered significant. The result was expressed as mean ±standard deviation.

CHAPTER FOUR

4.0 RESULTS

4.1Yield of Crude Extract and Phytochemical Content of the Extracts

The extraction of the plant materials yielded 14.432% of leaf used and 11.052% of root used

Phytochemical content of Leaf and Root extracts of *Ziziphus lotus*was carried out both qualitatively and quantitatively. The qualitative determination (Table 4.1) showed the presence of Saponin, Tannin, flavonoid, Alkaloid, Cyanogenicglycoside, Cardiac glycoside in both Leaf and Root extract

Quantitative determination of the phytochemical content of the plant extracts revealed variation in the quantity of the chemical present in the extract (Table 4.2). The root extract had higher phytochemicals than the leaf. Alkaloid, Saponin, and Tannin are higher in the root extract while flavonoid was higher in the leaf

Table4.1: Qualitative Phytochemical Determination of Methanol Crude Extract of Leaf and Root of *Zizipus lotus*

Phytochemicals	Leaves Extract	Root Extract
Flavonoids	+	+
Alkaloids	+	+
Tannins	+	+
Saponins	+	+
Cyanogenicglycosides	+	+
Cardiacglycosides	+	+

Table 4.2: Quantitative Determination of Phytochemicals content of Methanol Crude Extract of Leaf and Root of *Ziziphus lotus*

	Yield (%)	
Phytochemical	Leaf extract	Root Extract
flavonoids	12.14±0.70	10.08±0.84
Alkaloids	17.22±1.00	19.18±1.38
Tannins	8.02±1.40	13.62±0.67
Saponins	13.43±1.57	16.00±1.09
Cyanogenic glycosides	0.80±0.17	0.33±0.11
Cardiac glycosides	0.40±0.80	0.60±0.14

4.2 Antibiotic Susceptibility of the Test Bacteria.

The antibacterial susceptibility of the test bacteria is shown in (Table 4.3). Each of the bacteria was resistant to at least three out of the five antibiotic used. *S. aureus* was resistant to Erythromycin, tetracycline and amoxicillin but susceptible to Ciprofloxacin and Gentamycin. *Salmonella* species was resistant to Gentamycin, Erythromycin, and Amoxicillinbut susceptible to Ciprofloxacin and Tetracycline. *E. coli* was resistant to Erythromycin, Amoxicillin and Gentamycin, but susceptible to Ciprofloxacin and Tetracycline. *P. aeruginosa* was resistant to Erythromycin, Gentamycin and Tetracycline, but resistant to Amoxicillin and Ciprofloxacin. Therefore,the test bacteria are qualified to be multidrug resistant bacteria based on the CLSI (2010) criteria.

Table 4.3: Antibiotic susceptibility of Test Bacteria

Test Bacteria	Antibiotics (10µg)				
	ERY	**TET**	**AMX**	**GENT**	**CIPX**
S. aureus	–	–	–	+	+
Salmonella. Spp	–	+	–	–	+
E.coli	–	+	–	–	+
P. aeruginosa	–	–	–	+	+

Key: – Resistant

+ Susceptible

ERY= Erythromycin, TET= Tetracycline, AMX= Amoxicillin, GENT= Gentamycin, CIPX= Ciprofloxacin

4.3 Antibacterial Activity of Methanol Crude Extract of Leaf and Root of *Ziziphus lotus* on Multidrug Resistant Bacteria

Table 4.4shows the antibacterial activity of methanol crude extract of leaf at different concentrations (200, 100, 50, and 25 mg/ml) against the multidrug resistant bacteria. The increase in concentration of the extract caused increased zone of growth inhibition of the bacteria. The broad spectrum antibiotic (Ciprofloxacin 10μg) used as standard showed high zone of inhibition against *E.coli* and *P. aeruginosa* (30.33±3.00 mm) and least against *Salmonella* species (27.00±1.00 mm).The extract showed highest zone of inhibition (15.33±1.15 mm) against *S. aureus*, and leastagainst *E. coli* (10.33±1.15 mm) which is significantly different from the standard drug used.

Table 4.4: Antibacterial Activity of Methanol crude Extract of Leaf of *Ziziphus lotus* on Bacteria

	Zones of inhibition (mm)					
	Extract Concentration(mg/ml)					
Bacteria	Ciprofloxacin (10µg)	200	100	50	25	DMSO (5%)
S. aureus	29.33 ± 1.53^{f}	15.33 ± 1.15^{e}	12.00 ± 2.00^{d}	9.00 ± 1.73^{c}	7.67 ± 0.58^{b}	0.00 ± 0.00^{a}
Salmonella spp	27.67 ± 1.53^{e}	12.00 ± 1.00^{d}	9.00 ± 1.00^{c}	7.33 ± 0.58^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
E.coli	30.33 ± 2.52^{d}	10.33 ± 1.53^{c}	7.33 ± 0.58^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
P. aeruginosa	30.00 ± 3.00^{e}	11.00 ± 1.00^{d}	9.00 ± 1.00^{c}	7.67 ± 0.58^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

Values are Mean ± SD, of triplicate determination. Values with different superscript are significantly different within rows ($p<0.05$)

Table 4.5showed the antibacterial activity of methanol crude extract of root at different concentrations (200, 100, 50, and 25 mg/ml) against the test bacteria. Increase in concentration of the extract increased zone of growth inhibition of the test bacteria. The broad spectrum antibiotic of concentration 10µg (Ciprofloxacin 10µg) used as standard showed high zone of inhibition against *E.coli* and *P. aeruginosa* (30.33 ±3.00 mm) and least against *Salmonella* species (27±00 mm).The extractshowed highest zone of growth inhibition (16.33±1.53 mm) against *S. aureus*, and least against *E. coli* (11.67±1.53 mm) at 200 mg/ml and has no effect against *E. coli* and *P. aeruginosa*at concentration of 25mg/ml.

Table 4.5: Antibacterial Activity of Methanol crude Extract of Root of *Ziziphus lotus* on Bacteria

	Zones of inhibition (mm)					
	Extract Concentration(mg/ml)					
Bacteria	Ciprofloxacin (10µg)	200	100	50	25	DMSO (5%)
S. aureus	29.33±1.53^{f}	16.33±0.58^{e}	14.00±1.00^{d}	12.00±1.00^{c}	8.00±1.00^{b}	0.00±0.00^{a}
Salmonella spp	27.67±1.53^{e}	13.00±1.00^{d}	10.33±0.58^{c}	8.33±1.58^{b}	0.00±0.00^{a}	0.00±0.00^{a}
E.coli	30.33±2.52^{d}	11.67±1.53^{c}	8.33±1.53^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
P. aeruginosa	30.00±3.00^{e}	12.00±1.00^{d}	9.00±1.00^{c}	7.67±1.15^{b}	0.00±0.00^{a}	0.00±0.00^{a}

4.4 Antibacterial Activity of Ethyl acetate, n-hexane, n-butanol and Aqueous Fractions of the Extracts against the Test Bacteria

The fractions obtained from fractionation of crude extract were tested for activity. The n-hexane and ethyl acetate has no activity against the test bacteria (Table 4. 6) and (Table 4.7) respectively. The aqueous fraction has weak activity against *S. aureus* and *salmonella* spp at concentration of 200mg/ml (Table 4.8). The n- butanol has the best activity compare to the others (Table 4.9). From the result obtained n- butanol fraction of both leaf and root extract have activity, this indicated that the active compound is soluble in polar organic solvent.

Table 4.6: Antibacterial activity of n- hexane Fraction of Methanol Leaf Extract of *Ziziphus lotus* on Bacteria

	Zone of inhibition (mm)					
	Extract concentration (mg/ml)					
Bacteria	Ciprofloxacin 10µg	200	100	50	25	DMSO (5%)
S. aureus	29.33±1.53[b]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
Salmonella. Spp	27.33±1.53[b]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
E. coli	30.33±1.53[b]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
P. aeruginosa	30.00±1.00[b]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]

Values are Mean ± SD, of triplicate determination. Values with different superscripts are significantly different within rows ($p<0.05$)

Table 4.7: Antibacterial Activity of Ethyl acetate Fraction of Methanol Extract of Leafof *Ziziphus lotus*on Bacteria

	Zone of inhibition (mm)					
	Extracts concentration (mg/ml)					
Bacteria	Ciprofloxacin 10µg	200	100	50	25	DMSO (5%)
S.aureus	29.33±1.53[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00 ±0.00[a]	0.00 ±0.00[a]
Salmonella. Spp	27.33±1.53[a]	0.00 ±0.00[a]	0.00 ±0.00[a]	0.00 ±0.00[a]	0.00 ±0.00[a]	0.00±0.00[a]
E. Coli	30.33±1.53[a]	0.00±0.00[a]	0.00 ±0.00[a]	0.00 ±0.00[a]	0.00±0.00[a]	0.00 ±0.00[a]
P. aeruginosa	30.00±1.00[a]	0.00±0.00[a]	0.00 ±0.00[a]	0.00±0.00[a]	0.00 ±0.00[a]	0.00 ±0.00[a]

Values are Mean ± SD, of triplicate determination. Values with different superscripts are significantly different within rows ($p<0.05$)

Table 4.8: Antibacterial Activity of Aqueous fraction of Methanol Leaf Extract of *Ziziphus lotus* on Bacteria

	Zone of inhibition (mm)					
	Extracts concentration (mg/ml)					
Bacteria	Ciprofloxacin 10µg	200m	100	50	25	DMSO (5%)
S.aureus	29.33±1.53^{c}	10.00±1.00^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
*Salmonella.*Spp	27.33±1.53^{c}	8.00±1.53^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
E. coli	30.33±1.53^{b}	0.00±0.00^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
P.aeruginosa	30.00±1.00^{b}	0.00±0.00^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}

Values are Mean ± SD, of triplicate determination. Values with different superscripts are significantly different within rows ($p<0.05$)

Table 4.9: Antibacterial Activity of n- butanol Fraction of Methanol Leaf Extract of *Ziziphus lotus* on Bacteria

	Zone of inhibition (mm)					
	Extracts concentration (mg/ml)					
Bacteria	Ciprofloxacin (10µg)	200	100	50	25	DMSO (5%)
S. aureus	29.33±1.53^{f}	18.00±1.00^{e}	16.00±1.00^{d}	12.33±1.53^{c}	9.67±0.58^{b}	0.00±0.00^{a}
Salmonella spp	27.33±1.53^{f}	15.33±1.53^{e}	12.33±1.53^{d}	10.33±1.53^{c}	8.33±0.58^{b}	0.00±0.00^{a}
E.coli	30.33±1.53^{e}	13.67±0.58^{d}	11.00±1.00^{c}	7.67±0.58^{b}	0.00±0.00^{a}	0.00±0.00^{a}
P. aeruginosa	30.00±1.00^{f}	14.00±1.00^{e}	12.67±0.58^{d}	10.00±1.00^{c}	7.33±0.58^{b}	0.00±0.00^{a}

Values are Mean ± SD, of triplicate determination. Values with different superscripts are significantly different within rows ($p<0.05$)

. Table 4.10: Antibacterial Activity of n- hexane Fraction of Methanol Root Extract of *Ziziphus lotus* on Bacteria

Bacteria	Zone of inhibition (mm)					
	Extracts concentrations (mg/ml)					
	Ciprofloxacin 10µg	200	100	50	25	DMSO (5%)
S. aureus	29.33±1.53^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00 ±0.00^{a}
Salmonella. Spp	27.33±1.53^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
E. coli	30.33±1.53^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
P. aeruginosa	30.00±1.00^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}

Values are Mean ± SD, of triplicate determination. Values with different superscripts are significantly different within rows ($p<0.05$)

Table 4.11: Antibacterial Activity of Ethyl acetate Fraction of Methanol Root Extract of *Ziziphus lotus* on Bacteria

	Zone of inhibition (mm)					
	Extracts concentration (mg/ml)					
Bacteria	Ciprofloxacin 10µg	200	100	50	25	DMSO (5%)
S. aureus	29.33±1.53^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
*Salmonella.*Spp	27.33±1.53^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
E. coli	30.33±1.53^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
P. aeruginosa	30.00±1.00^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}

Values are Mean ± SD, of triplicate determination. Values with different superscripts are significantly different within rows ($p<0.05$)

Table 4.12: Antibacterial Activity of Aqueous fraction of Methanol Root Extract of *Ziziphus lotus* on Bacteria

	Zone of inhibition (mm)					
	Extracts concentration (mg/ml)					
Bacteria	Ciprofloxacin 10µg	200	100	50	25	DMSO (5%)
S. aureus	29.33±1.53^{d}	12.00±1.58^{c}	8.00±1.00^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
*Salmonella.*Spp	27.33±1.53^{c}	9.00±0.58^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
E. coli	30.33±1.53^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
P. aeruginosa	30.00±1.00^{b}	0.00 ±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}

Values are Mean ± SD, of triplicate determination. Values with different superscripts are significantly different within rows ($p<0.05$)

Table 4.13: Antibacterial Activity of n butanol Fraction of Methanol Root Extract of *Ziziphus lotus* on Bacteria

Bacteria	Zone of inhibition (mm)					
	Extracts concentrations (mg/ml)					
	Ciprofloxacin 10µg	200	100	50	25	DMSO (5%)
S. aureus	29.33±1.53^{f}	22.00±2.00^{e}	18.33±0.58^{d}	15.33±0.58^{c}	12.33±0.58^{b}	0.00±0.0^{a}
S*almonella.* spp	27.33±1.53^{f}	18.33±0.58^{e}	15.00±1.00^{d}	11.00±1.00^{c}	9.33±1.00^{b}	0.00±0.00^{a}
E. coli	30.33±1.53^{e}	15.33±1.53^{d}	11.00±1.00^{c}	8.00±1.00^{b}	0.00±0.00^{a}	0.00±0.00^{a}
P. aerruginosa	30.00±1.00^{f}	16.00±1.00e	13.00±1.00^{d}	11.00±1.00^{c}	8.00±1.00^{b}	0.00±0.00^{a}

Values are Mean ± SD, of triplicate determination. Values with different superscripts are significantly different within row ($p<0.05$)

4.5 Antibacterial Activity of Partially purified n-butanol Fractions of Leaf and Root of *Ziziphus lotus* against Bacteria Isolates.

The n-butanol fractions of both leaf and root extract of *Ziziphus lotus* were partially purified using column chromatography. Each of the fractions gave total number of forty eight sub fractions. The sub fractions were pooled together based on their TCL result to form four fractions. Those with spots that have similar R_f values were combined to form one fraction. The pooled fractions were labeled A_L to D_L for leaf and A_R to D_R for root. Fraction B of leaf (B_L) and fraction B of root (B_R) have better activities compare to others (Table 4.15) and (Table 4.19) respectively.

Table 4.14: Antibacterial Activity of partially purified Fraction A of the Methanol Leaf Extract (A_L) on Bacteria

	Zone of inhibition (mm)					
	Extracts Concentrations (mg/ml)					
Bacteria	Ciprofloxacin 10µg	200	100	50	25	DMSO (5%)
S. aureus	29.00±1.00e	14.00±1.00^{d}	11.00±1.00^{c}	7.67±0.58^{b}	0.00±0.00^{a}	0.00±0.00^{a}
*Salmonella*spp	22.00±1.00^{d}	12.00±1.00^{c}	8.00±1.00^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
E. coli	30.00±1.00^{c}	9.00±1.00^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
P. aeruginosa	30.00±2.00^{d}	10.67±1.53^{c}	8.00±1.00^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}

Values are Mean ± SD, of triplicate determination. Values with different superscripts are significantly different within rows ($p<0.05$)

Table 4.15: Antibacterial Activity of Partially Purified Fraction B of the Methanol Leaf Extract (B_L**)** on Bacteria

	Zone of inhibition (mm)					
	Extracts Concentration (mg/ml)					
Bacteria	Ciprofloxacin 10µg	200	100	50	25	DMSO (5%)
S. aureus	29.33±1.53^{f}	21.00±1.00^{e}	19.00±1.00^{d}	16.00±1.00^{c}	12.00±1.00^{b}	0.00±0.00^{a}
Salmonella. Spp	27.33±1.53^{f}	18.00±1.00^{e}	14.33±0.58^{d}	11.00±1.00^{c}	9.00±1.00^{b}	0.00±0.00^{a}
E. coli	30.33±1.53^{f}	15.33±0.58^{e}	12.00±1.00^{d}	10.33±0.58^{c}	7.33±0.58^{b}	0.00±0.00^{a}
P. aeruginosa	30.00±1.00^{f}	17.00±1.00^{e}	14.00±1.00^{d}	11.00±1.00^{c}	8.33±0.58^{b}	0.00±0.00^{a}

Values are Mean ± SD, of triplicate determination. Values with different superscripts are significantly different within rows ($p<0.05$)

Table4.16: Antibacterial Activity of partially purified Fraction C of Methanol Leaf Extract (C_L) on Bacteria

	Zone of inhibition (mm)					
	Extracts concentrations (mg/ml)					
Bacteria	Ciprofloxacin 10µg	200	100	50	25	DMSO (5%)
S. aureus	29.00±1.00f	16.00±1.00^{e}	13.00±1.00^{d}	10.00±1.00^{c}	7.33±0.57^{b}	0.00±0.00^{a}
Salmonella. Spp	22.00±1.00d	10.33±1.00^{c}	8.33±1.00^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
E. coli	30.00±1.00^{c}	9.33±1.53^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
P. aeruginosa	30.00±2.00^{d}	10.33±1.53^{c}	7.66±0.58^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}

Values are Mean ± SD, of triplicate determination. Values with different superscripts are significantly different within rows ($p<0.05$

Table 4.17: Antibacterial Activity of partially purified Fraction D of Methanol Leaf Extract (D_L) on Bacteria

	Zone of inhibition (mm)					
	Extracts Concentration (mg/ml)					
Bacteria	Ciprofloxacin 10µg	200	100	50	25	DMSO (5%)
S. aureus	29.00±1.00^{d}	10.33±1.53^{c}	8.33±0.58^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
*Salmonella*spp	22.00±1.00^{c}	9.00±1.00^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
E. coli	30.00±1.00^{c}	7.67±1.00^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
P. aeruginosa	30.00±2.00^{c}	9.00±1.00^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}

Values are Mean ± SD, of triplicate determination. Values with different superscripts are significantly different within rows ($p<0.05$)

Table4.18: Antibacterial Activity of Partially Purified Fraction A of the Methanol Root Extract (A_R) on Bacteria

	Zone of inhibition (mm)					
	Extracts concentrations (mg/ml)					
Bacteria	Ciprofloxacin 10µg	200	100	50	25	DMSO (5%)
S. aureus	29.00±1.00^{e}	16.00±1.00^{d}	12.00±1.00^{c}	8.67±0.58^{b}	0.00±0.00^{a}	0.00±0.00^{a}
Salmonella spp	22.00±1.00^{d}	14.00±1.00^{c}	9.00±1.00^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
E. coli	30.00±1.00^{c}	12.33±0.58^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
P. aeruginosa	30.00±2.00^{d}	13.67±1.53^{c}	9.00±1.0^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}

Values are Mean ± SD, of triplicate determination. Values with different superscripts are significantly different within rows ($p<0.05$)

Table 4.19: Antibacterial Activity of Partially Purified Fraction B of the Methanol Root Extract (B_R) on Bacteria

	Zone of inhibition (mm)					
	Extracts concentration (mg/ml)					
Bacteria	Ciprofloxacin 10µg	200	100	50	25	DMSO (5%)
S. aureus	29.33±1.53^{f}	23.33±1.53^{e}	21.00±1.00^{d}	17.00±1.00^{c}	14.00±1.00^{b}	0.00±0.00^{a}
Salmonella Spp	27.33±1.53^{f}	20.00±1.00^{e}	16.33±1.53^{d}	13.00±1.00^{c}	10.00±1.00^{b}	0.00±0.00^{a}
E. coli	30.33±1.53^{f}	17.33±1.53^{e}	14.00±1.00^{d}	11.00±1.00^{c}	7.33±0.58^{b}	0.00±0.00^{a}
P. aeruginosa	30.00±1.00^{f}	19.00±1.00^{e}	16.00±1.00^{d}	13.00±1.00^{c}	10.00±1.00^{b}	0.00±0.00^{a}

Values are Mean ± SD, of triplicate determination. Values with different superscripts are significantly different within rows ($p<0.05$)

Table 4.20: Antibacterial Activity of Partially Purified fraction C of methanol root extracts (C_R) on Bacteria

	Zone of inhibition (mm)					
	Extracts concentration (mg/ml)					
Bacteria	Ciprofloxacin 10µg	200	100	50	25	DMSO (5%)
S. aureus	29.00±1.00^{f}	18.00±1.00^{e}	15.33±1.50^{d}	11.33±0.57^{c}	7.66±0.57^{b}	0.00±0.00^{a}
Salmonella Spp	22.00±1.00^{d}	13.00±1.00^{c}	9.00±1.00^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
E. coli	30.00±1.00^{c}	10.33±1.53^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
P. aeruginosa	30.00±2.00^{d}	12.66±1.53^{c}	8.66±0.58^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}

Values are Mean ± SD, of triplicate determination. Values with different superscripts are significantly different within rows ($p<0.05$)

Table 4.21: Antibacterial Activity of Partially Purified Fraction D of Methanol Root Extract (D_R) on Bacteria

	Zone of inhibition (mm)					
	Extracts Concentrations (mg/ml)					
Bacteria	Ciprofloxacin 10µg	200	100	50	25	DMSO (5%)
S. aureus	29.00±1.00^{d}	13.33±1.53^{c}	8.67±0.58^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
S*almonella* spp	22.00±1.00^{c}	10.00±1.00^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
E. coli	30.00±1.00^{c}	9.00±1.00^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
P. aeruginosa	30.00±2.00^{c}	10.00±1.00^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}

Values are Mean ± SD, of triplicate determination. Values with different superscripts are significantly different within rows ($p<0.05$)

4.6 Identification of Compounds using GC-MS

The GC-MS result revealed the presence of Quinoline, Hexanoic acid undecyl ester, Oleic acid, Octadec-9-noic acid and 6-Octadecenoic acid in the root extract. And Quinoline, 6-Octadecenoic acid, Floxuridine and 3-Eicosene in the leaf extract (Table 4.22).These could possibly contribute to the activity of the plant extracts.

Table 4.22 Compounds identified by GC-MS analysis of the extract

S/No	Retention Time	Compound	Molecular formula	Molecular weight(g/mol)
1	49.985	Octadec-9-enoic acid	$C_{18}H_{34}O_2$	282.46
2	48.641	Hexanoic acid undecylester	$C_{17}H_{12}O_3$	144.17
3	49.080	3-Eicosen	$C_{20}H_{40}$	280.53
4	49.038	Floxuridine	$C_9H_{11}FN_2O_5$	249.19
5	50.958	6- Octadecenoic acid	$C_{18}H_{34}O_2$	286.00
6	49.152	Oleic acid	$C_{19}H_{36}O_2$	294.00
7	46.690	Quinoline	C_9H_7N	129.00

4.7 Minimum Inhibitory Concentration (MIC) of Crude Extract and Partially Purified Fractions.

Table 4.23 showed the minimum inhibitory concentration (MIC) of both the extract and fraction of both leaf and root on the test bacteria

The minimum inhibitory concentration of crude extract and partially purified fractions was determined. From the result the extract has more activity against *S. aureus* with minimum inhibitory concentration of 12.50 mg/ml for the crude extract and 3.13 mg/ml for the fraction.*Salmonella* species has MIC of 25 mg/ml for the crude extracts and 6.25 mg/ml for the fractions. *E.coli* has 25 mg/ml for the crude extracts and 12.50 mg/ml for the fraction,*P. aeruginosa* has 25 mg/ml for the crude extracts and 6.25 mg/ml for the root fraction and 12.50 mg/ml for the leaf fraction.

Table 4.23: Minimum Inhibitory Concentration of the crude Extract and Fraction (MIC)

Type of Extract	Bacteria			
	S. aureus	*Salmonella*. Spp	*E. coli*	*P. aeruginosa*
	Extracts concentration (mg/ml)			
Crude Extract of root	12.50	25.00	25.00	25.00
Crude Extract of leaf	12.50	25.00	25.00	25.00
Fraction B_R	3.13	6.25	12.50	6.25
Fraction B_L	3.13	6.25	12.50	12.50

B_R is the fraction of root extract obtained after purification while B_L is for that of the leaf extract

4.8 Minimum Bactericidal Concentration (MBC) of both Crude Extract and Partially Purified Fraction of Root and Leaf.

Table 4.24 showed the minimum bactericidal concentration of both the crude and fractions of leaf and root.

Minimum inhibitory concentration (MBC) of both the crude extract and partially purified fractions was determined. The result showed that *S. aureus* has the lowest MBC of 25mg/ml for the crude extract and 6.25mg/ml for the fractions. Followed by *Salmonella* species and *P.aeruginosa* with MBC of 50mg/ml for crude extract and 12.50mg/ml for the fraction, then *E.coli* has MBC of 50mg/ml for the crude and 25mg/ml for the fractions.

Table 4.24: Minimum Bactericidal Concentration (MBC) of both the Extracts and Fractions

	Bacteria			
	S. aureus	*Salmonella.* Spp	*E. coli*	*P. aeruginosa*
Type of Extract	Extracts concentrations (mg/ml)			
Crude Extract of root	25	50	50	50
Crude Extract of leaf	25	50	50	50
Fraction B_R	6.25	12.50	25	12.50
Fraction B_L	6.25	12.50	25	25

B_R is the fraction of root extract obtained after purification while B_L is for that of the leaf extract.

CHAPTER FIVE

5.0 DISCUSSION

Infectious diseases are one of the major causes of mortality and morbidity worldwide especially in developing countries(Silva and Fernandes 2010).They constitute the second leading cause of death worldwide (El Zawahry *et al.,* 2013).

Treatment of infectious disease is still a challenge in the health sector because of several side effects of some synthetic drugs and resistance posed by most of the organism that cause this disease. These Organisms are resilient and developed resistance to most of the conventional antibiotics (El Zawahry *et al.,* 2013)

Multi drug resistance is a condition where by the organism is no longer sensitive to at least three different antibiotics from different classes of antibiotics (CLSI 2010). Multidrug resistance is at the increase among the pathogenic organisms especially bacteria worldwide. This condition has raised an urgent need to search for an alternative to fight drug resistance. Research has turned to natural source to search for new, safer and more potent antibiotics that can combat drug resistance. The results of different studies provide evidence that some medicinal plants might indeed be potential source of new antibacterial agents (Hidayathulla *et al.,* 2011). Plants are important source of potentially useful chemical compound structures for the development of new chemotherapeutic agents. The first step towards this goal is *in vitro* antibacterial activity. The extracts of higher plant can be very good source of antibiotics against various bacterial pathogens. Plant based antimicrobial compounds have enormous therapeutic potentials as they can serve the purpose without any side effects that are often associated with synthetic antibacterial compounds (Hidayathulla *et al.,* 2011).

The presence of various phytochemicals seen in the extracts agreed with the work reported by (Naili*et al.,* 2010) and (Baktoaui *et al*,, 2014). They reported the presence of this compound in the leaf and fruit of *Ziziphus lotus* respectively. Phytochemicals have been reported to exhibit various biological activities in plants and human health. Antimicrobial activity of plant extract has been attributed to some of these phytochemicals as it was reported by (Hidayathulla *et al.,* 2011). The variation of concentration of phytochemicalcs in the two extracts could be as a result of the distribution of the compounds in different parts of the plants.

The activity of the crude extracts against the bacteria was dose dependent and it was observed to be better against *S. aureus* and least against *E. coli*. This finding is in line with work carried out on methanol extract of leaf of the same plant by (Naili *et al.,* 2010) who reported high activity of the extract against *S. aureus* and low against *E. coli, Pseudomonas aeruginosa* and *Salmonella* species.(Rsaissi *et al.,* 2013) also observed the same in a separate work done on the fruit of the same plant. The slight difference observed in the zone of inhibition between the leaf and root extract could be as a result of the quantity of phytochemical distributed in the plant parts. The lack of activity of n-hexane and ethyl acetate fraction could be as a result of non polarity of the solvents which could not dissolve the active compounds present in the fractions. The n-butanol fractions that showed good activity against the test bacteria could be attributed to the polar nature of the solvent which dissolved the active compounds from the extracts. This variation in the activity of the fractions showed that the active compounds could be more soluble in organic polar solvent. Hence, n-butanol could be a better solvent to extract the plant materials. This finding agreed with the report of (Nwodo *et al.*, 2010) who observed that fractionation of extract can lead to increase or loss of activity. (Hemraj and Jindal 2012) also reported that methanol extract of *Psidiumguajava* has better activity against resistant strain of *S. aureus*,

Salmonella species, and *E. coli* compared to that of n- hexane and ethyl acetate.The better activity of the partially purified fraction B of both leaf and root compare to other fractions could be as a result of polarity of solvent system used for the purification. This also agreed with the report of (Nwodo *et al.*, 2010).

The presence of the compounds identified by GC-M S analysis of the fraction could also contributed to the activity of the extracts against the test bacteria. This result is similar to the report of (Ashraf *et al* 2015) on the leaf extract of the same plant. It can also be supported by the work carried out by (Beammar *et al* 2010: Nazif 2002).The MIC and MBC values for the partially purified fraction (Table4.23) and (Table 4.24) was better compare to that of the crude extracts. This indicated that purification of the extract increases the activity of the compound.

Generally, this study reports the efficacy of extract of leaf and root of *Ziziphus lotus* on resistant strain of *S. aureus*, *Salmonella* species, *E. coli* and *P. aeruginosa*. From the fact that the study report resistance of the test bacteria to some antibiotics, the extract from this plant has potential to offer effective, less expensive treatment option to diseases cause by these test bacteria. Throughout the study the activity of the extracts has been higher against *S. aureus* than other bacteria. This is due to the fact that plant extracts are more effective against gram positive bacteria than gram negative (Suffredini *et al.*, 2006). This observation could be explained with reference to the cellular structure of gram positive and gram negative bacteria. The gram positive bacteria do not have outer membrane that can possibly hinder the entrance of the bioactive compounds to locate the target. But the gram negative bacteria have outer membrane with lipopolysaccharide that can possibly hinder the entrance of the bioactive compounds to locate the target. Contrast to our result, (Abalaka *et al* 2010) reported large zone of inhibition by *Ziziphus muritiana* leaf extract against *E. coli* compared to other gram positive bacteria. This antibacterial

activity of the extracts against the test bacteria could be attributed to the presence of the compounds in the extracts. This result supports the use of this plant in traditional medicine for treatment of infectious diseases and development of antibiotic from the plant.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

From the result of this finding methanolic extract of root of *Ziziphus lotus* has higher content of Alkaloids, Saponins and Tannins than the leaf extract. n- butalnol fractions of the extracts have better activity against Resistant strain of *E. coli, P. aeruginosa, Salmonella* spp, and *S. aureus* compare to n hexane, ethyl acetate, and aqueous fractions. Hence n butanol could be a better solvent for extraction of this compound. The plant extract has better activity against *S. aureus* than the other test bacteria. Methanolic extracts of leaf and root of *Ziziphus lotus* has antibacterial activity against resistant strain of *E. coli, P. aeruginosa, Salmonella* spp, and *S. aureus*. This finding validates the use of this plant in traditional medicine and the plant could be a good source to develop new antibiotics to fight bacterial infection.

6.2 RECOMMENDATIONS

From the result of this finding the followings should be carried out on the plant extract.

i. Toxicity study should be carried out on the plant extracts to ascertain the safety of the extract
ii. In vivo study should be carried out using the plant extracts to see the efficacy of the extract in animal model
iii. The extract of this plant should be tested against other organism like protozoa, Helminthes and fungi
iv. Further study should be carried out to isolate the active compound for pharmaceutical and industrial use.
v. Mechanism of action of the compound should also be studied.

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