ANTIMICROBIAL ACTIVITY OF METHANOLIC AND AQUEOUS EXTRACTS AND AMINO ACID PROFILE OF THE PARTIALLY PURIFIED PROTEIN OF *GUIERA SENEGALENSIS* (MOSHI MEDICINE)

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OCTOBER, 2015

DECLARATION

I declare that the work in this Dissertation entitled "Antimicrobial activity of methanolic and aqueous extracts and amino acid profile of the partially purified protein of *Guiera senegalensis* (moshi medicine)*"* has been carried out by me in the Department of Biochemistry, Faculty of Science. The information derived from literature has been dully acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree at this or any other institution.

Jiyil Markus Kirwe

Name of Student	Signature	Date

CERTIFICATION

This Dissertation entitled "ANTIMICROBIAL ACTIVITY OF METHANOLIC AND AQUEOUS EXTRACTS ANDAMINO ACID PROFILE OF THE PARTIALLY PURIFIED PROTEIN OF *GUIERA SENEGALENSIS* (MOSHI MEDICINE) " BY KIRWE MARKUS JIYIL meets the regulations governing the award of the degree of MSc Biochemistry of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

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ABSTRACT

Microorganisms have evolved defence mechanisms against antimicrobial agents and are resistant to some antibiotics.This study was aimed at evaluating the antimicrobial activity of methanolic and aqueous extracts and amino acid profile of the partially purified protein of *Guiera senegalensis* (moshi medicine). The antimicrobial activities of the extracts were assayed by agar disc diffusion and nutrients broth dilution techniques. Antimicrobial activity showed that, all the extracts were active against most of the isolates except *Candida albican* (fungus). Aqueous extract of matured and young leaves showed zones of inhibition ranging from 11.00 -26.00mm while aqueous extract of matured and young roots showed zones of inhibitions ranging from 11.00 – 19.00mm. Methanolic of matured and young leaves showed zones of inhibition ranging from 11.00 – 42.00mm while matured and young roots ranging from 11.00 – 37.00mm. The crude proteins were active against gram positive bacteria. Maximum zone of inhibition (42.00±1.00mm) was observed in methanolic extracts of young leaves against *Staphylococcus aureus* at 100mg/ml. Most extracts exhibited minimum inhibitory concentration (MIC) at range of 6.25mg/ml and 12.5mg/ml and MBC at 12.5mg/ml and 25mg/ml. The methanolic extract was observed to be more potent than the aqueous extract. The young leaves and roots were more active than matured leaves and roots of the plant. Seventeen (17) amino acids were quantified, indicating high Concentration in young leaves than matured leaves and roots. Glutamic acids and Aspartic acid were found in higher concentration in both leaves and roots of the plant. The molecular weight of the partial purified proteins of the matured leaves were 25.67 kDa and 149.2 kDa at protein concentrations of 1.10mg/ml while the young leaves were 20.33 kDa and 45.50 kDa at protein concentration of 1.20mg/ml. The presence of bioactive secondary metabolites, antimicrobial amino acids, low minimum inhibitory concentration (MIC) and minimum bacteriocidal concentration (MBC) justifies the traditional uses of the leaves and roots of *Guiera senegalensis* for therapeutic purposes.

Table of Contents

Page

Title Page ---------- iii

Declaration ---------- iv

Certification---------- v

Acknowledgement ---------- vi

Dedication---------- viii

Abstract---------- ix

Table of Contents ---------- x

List of Figures---------- xvi

List of Tables---------- xvii

List of Plates ----------xix

List of Appendices---------- xx

Abbreviations ---------- xxi

CHAPTER ONE----------1

1.0 INTRODUCTION ---------- 1

1.1 Background ----------1

1.2 Statement of the Problem ---------- 5

1.3 Justification for the Study----------6

1.4 Null Hypothesis----------6

1.5 Aim and Objectives ----------7

1.5.1 Aim of the study----------7

1.5.2 Specific objectives----------7

CHAPTER TWO---------- 8

2.0 LITERATURE REVIEW ---------- 8

2.1 Traditional Medicine ---------- 8

2.2 Drug Discovery from Plant----------10

2.3 The Plant *Guiera senegalensis* ---------- 12

2.3.1 Taxonomy----------12

2.3.2 Common names----------12

2.3.3 Botanical description ----------15

2.3.4 Ecology and distribution ----------15

2.3.5 Propagation and planting ----------15

2.4.6 Disease and pest---------- 15

2.3.7 Use of *G. senegalensis*---------- 16

2.4 Amino Acids, Peptides and Proteins----------17

2.4.1 Amino acid profile----------18

2.5 Antimicrobial Proteins---------- 18

2.5.1 Characteristic structures of antibacterial peptides---------- 20

2.5.2 Mechanisms by which microorganisms exhibit resistance to antimicrobials ---------- 22

2.5.3 Mechanism of action of antimicrobial agents---------- 23

2.5.3.1 *Inhibition of synthesis of cell wall peptidoglycan*----------24

2.5.3.2 *Inhibition of the nucleic acid synthesis*---------- 24

2.5.3.3 Inhibition of protein synthesis----------25

2.5.3.4 Disruption of cell membrane---------- 26

2.5.3.5 Inhibition of metabolic activities---------- 26

2.6 Antimicrobial Agents---------- 29

2.6.1 Antimicrobial properties of medicinal plants---------- 30

2.6.2 Antibacterial----------31

2.6.3 Antifungal----------31

2.6.4 Antiviral----------32

2.7 An Overview of Test Organisms ----------34

2.7.1 *Staphylococcus aureus*----------34

2.8.2 *Bacillus subtilis*---------- 34

2.7.3 *Escherichia coli* ---------- -35

2.7.4 *Salmonella typhimurium*---------- ---.36

2.7.5 *Candida albicans*---------- ---37

CHAPTER THREE----------38

3.0 MATERIALS AND METHODS ---------- 38

3.1 Materials----------38

3.1.1 Sample collection ---------- 38

3.2 Methods----------**38**

3.2.1 Preparation of extracts ---------- 38

3.2.2 Methanol extraction ---------- .38

3.2.3 Cold water extraction ----------.39

3.2.4 Preparation of stock solution of extracts ----------39

3.2.5 Preparation of test organisms----------39

3.2.6 Determination of preliminary antimicrobial activity of extracts----------40

3.2.7 Agar well diffusion assay----------40

3.2.8 Determination of minimum inhibitory concentration (MIC) ----------41

3.2.9 Determination of minimum bactericidal concentration (MBC) ---------- 42

3.2.10 Partial purification of pntimicrobial protein/peptides----------42

3.2.11 Antimicrobial assay of crude and partial purified of protein/peptides----------43

3.2.12 SDS-PAGE of partial purified proteins----------43

3.2.13 Determination of amino acid profile---------- 43

3.2.14 Nitrogen determination----------44

3.2.15 Defatting sample---------- 45

3.2.16 Hydrolysis of the sample---------- 45

CHAPTER FOUR----------47

4.0 RESULTS----------47

4.1 Antimicrobial Screening of *G. senegalensis*----------

4.2 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) ---------- 63

4.3 Antimicrobial Screening of Crude and Protein Fractions. ---------- 66

4.4 Amino Acid Profile of the leaves and roots of *Guiera senegalensis*---------- 70

CHAPTER FIVE---------- 72

5.0 DISCUSSION ---------- 72

CHAPTER SIX---------- 78

6.0 CONCLUSION AND RECOMMENDATIONS ----------78

References ---------- 80

Appendices ----------93

List of Figures

Figure **Tile** **Page**

2.1. Matured *Giuera senegalensis* Plant…….. 13

2.2 . Young *Guiera senegalensis*…….. 14

2.3. An overview of AMP membrane permeability models……. 27

4.4 SDS PAGE of Young and Matured Leaf of *Guiera senegalensis*……...69

List of Tables

Table **Title** **Page**

2. 1. Plants processing Antimicrobial Activity.---------- 33

4.1 Antimicrobial screening of different concentration of methanol extract of matured leaf of *Guiera senegalensis*. ---------- 48

4.2 Antimicrobial screening of different concentration of methanol extracts of young leaves of *Guiera senegalensi* ----------50

4. 3 Antimicrobial screening of different concentration of methanol extract of matured root of *Guiera senegalensis.*---------- 52

4.4 Antimicrobial screening of different concentration of methanol extract of young root of *Guiera senegalensis.*---------- 54

4.5 Antimicrobial screening of different concentration of aqueous extract of matured leaves *Guiera senegalensis*---------- 56

4.6 Antimicrobial screening of different concentration of extract of young leaf of *Guiera senegalensis*.----------58

4. 7 Antimicrobial screening of different concentration of aqueous extract of matured root of *Guiera senegalensis.*----------60

4.8 Antimicrobial screening of different concentration of aqueous extract of young roots of *Guiera senegalensis.*---------- 62

4.9 Mininmum inhibitory concentration (MIC) of methanolic and aqueous extract of leaves and roots of *Guiera senegalensis.*----------64

4.10 Minimum bacteriocidal concentration (MBC) of methanolic and aqueous extract of leaves and roots of *Guiera senegalensis*----------65

4.11 Purification of Bioactive protein the leaves of young and matured *G. senegalensis.*- 67

4.12 Antimicrobial activity of the leaves and roots of the crude and partial purified protein fractions of *Guiera senegalensis*----------68

4.13 Amino Acids Profile of the leaves and roots of young and matured *G. senegalensis.* -- 71

List of Plates

Plate **Title** **Page**

1: Antimicrobial cultured plates of the leaves and roots extracts of *Guiera senegalensis*…….99

2: Plate of matured(3) and young (4) leaves of methanolic extracts that showed maximum zones of inhibition against *Staphylococcus aureus*……..100

3: Plate of the most active protein fractions of young (3) and matured (4) leaves of *G. senegalensis* that showed maximum zone of inhibition against *Staphylococcus aureu*…….101

List of Appendices

Appendix **Title** **Page**

i. Antimicrobial cultured plates of the leaf and root of extracts of *G. senegalensis*……...…93

ii. Zone of inhibition of methanol extract(susceptibility).....94

iii. Zone of inhibition of protein fractions of *G. senegalensis*…….……...…95

iv. Protein purification profile of matured leaf of *G. senegalensis*…….…….....96

v. Protein purification profile of young leaf of *G. senegalensis*……..……...97

vi. Protein purification profile of matured root of *G. senegalensis*……98

vii. Protein purification profile of young root of *G. senegalensis*…….……99

viii Standard Calibration Curve for quantitative determination of proteins…… 100

ix Graph of amino acids profile of matured leaf of *Guiera senegalensis*……..101

x Graph of amino acids profile of young leaf of *Guiera senegalensis*……....102

xi Graph of amino acids profile of matured root of *Guiera senegalensis*……..103

xii Graph of amino acids profile of young root of *Guiera senegalensis*……104

ABBREVIATION

AA Amino Acid

ABU Ahmadu Bello University

AMPs Antimicrobial Peptides

AOAC Association of analytical chemist

AU Arbitrary Unit

CAM Complementary Alternative Medicine

CCL4 Tetra chloromethane

CNS Central Nervous System

E.coli Escherichia coli

HIV Human Immuno Deficiency Virus

IgM Immunoglobulin M

LPS Lipopolysaccharides

MBC Minimum Bactericidal Concentration

MDR Multi-Drug Resistance

MIC Minimum Inhibitory Concentration

MRSA Methicillin Resistant Staphylococcus aureus

NE Norleucine equivalent

PABA Para- amino benzoic acid

PR Poline – Rich

SDS-PAGE Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

TSM Technicon Sequential Multi-Sample Amino Acid Analyzer

UTI Urinary Tract Infection

WHO World Health Organization

y-PGA y-Polyglutamic Acids.

CHAPTER ONE

INTRODUCTION

1.1 Background

Infectious diseases are the world's leading cause of premature deaths, killing almost 50,000 people every day. Infections due to a variety of bacterial etiologic agents, such as pathogenic *Escherichia coli, Vibrio cholerae, Areomonas spp., Shigella spp., Salmonella spp., Pseudomonas spp*., *Klebsiella spp*., *Campylobacter spp* , *Bacillus subtilis* and *Staphylococcus aureus* are most common. In recent years, drug resistance to human pathogenic bacteria has been commonly reported from all over the world (Hancock *et al*., 2012). Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases.One approach is to screen local medicinal plants for possible their antimicrobial properties.

Plant materials remain an important resource to combat serious diseases in the world. According to WHO (1993), 80 % of the world's population is dependent on the traditional medicine and a major part of the traditional therapies involves the use of plant extracts or their active constituents. Yet a scientific study of plants to determine their antimicrobial active compounds is a comparatively new field. The traditional medicinal methods, especially the use of medicinal plants, still play a vital role to cover the basic health needs in the developing countries.Antibiotic resistance among pathogenic bacteria is increasing at an alarming rate, and at the same time, few new antibiotics reach the market. Today, resistant and multi-drug resistant (MDR) pathogenic strains are widespread facing bigger problems when treating many common bacterial infections (Djeussi *et al.*, 2013).

Thus, antibiotic resistant bacteria may keep people sick longer, and sometimes people are unable to recover at all. Because of the concern about the side effects of conventional medicine, the use of natural products as an alternate to conventional treatment in healing and treatment of various diseases has been on the rise in the last few decades (Krishnaiah *et al.*,2009).This result to the development of antimicrobial peptides and other secondary metabolite for treatment of bacterial, virus and fungi.

Antimicrobial peptides (AMPs), are important part of the innate immune system, made up of small molecules that may present antibacterial, antifungal, antiparasitic, and antiviral activity (Hancock *et al.*, 2012). Usually these molecules are composed of 10–50 amino-acid residues, and arranged in different groups depending on the amino-acid composition, size, and conformation (Nakatsuji and Gallo , 2012).Antimicrobial peptides (AMPs) are found in most organisms. Apart from having an immune modulatory role, they also function in the protection against microbes (Wang, 2014). AMPs have promising therapeutic properties: they kill microbes rapidly, have broad activity-spectra and there are few reports of emerging bacterial resistance, and therefore much effort is focused on finding potential novel antibacterial drugs among AMPs .Medicinal plants have been identified and used throughout human history. Plants have the ability to synthesize a wide variety of chemical compounds that are used to perform important biological functions, and to defend against attack from predators such as insects, fungi and herbivorous mammals. At least 12,000 such compounds have been isolated so far; a number estimated to be less than 10 % of the total. Chemical compounds in plants mediate their effects on the human body through processes identical to those already well understood for the chemical

compounds in conventional drugs; thus herbal medicines do not differ greatly fro8pm conventional drugs in terms of how they work. (Cederlund *et al.*, 2011).

The continuous uses of antibiotic micro-organisms have become resistant to commonly used antibiotics, necessitating the search of new antimicrobial agents.This has created immense clinical problem in the treatment of infections diseases This enables herbal medicines to be as effective as conventional medicines, but also gives them the same potential to cause harmful side effects.

During the last century, the practice of herbals became the mainstream throughout the world. In spite of the great advances observed in modern medicine, plants still make an important contribution to healthcare. This is due to the recognition of the value of traditional medicine. Traditional medicine is a comprehensive term used to refer to both traditional medicine systems such as traditional Chinese medicine, and to various forms of indigenous medicine (WHO, 2002). *G. senegalensis* is being used in traditional medicine for the remedy of many ailments/diseases.The leaves are widely used for pulmonary and respiratory diseases, for coughs, as a febrifuge, colic and diarrhea, syphilis, beriberi, leprosy, impotence, rheumatism, diuresis, dysentery, gastrointestinal pain and disorder, rheumatism and fever. In addition, partially purified anthocyanin fraction from leaf extract of *G. senegalensis* has been shown to possess antioxidant property against CCl_4 induced oxidative stress in rats (Sule *et al.*,2011).Plant extracts contain a mixture of secondary metabolites including alkaloids, flavonoids, terpenoids, and other phenolic compounds; these molecules are associated to defense mechanisms of plants by their repellent or attractive properties, protection against biotic and abiotic stresses, and maintenance of structural integrity of plants.

Many commercially proven drugs used in modern medicine were initially used in crude form in traditional or folk healing practices, or for other purposes that suggested potentially useful biological activity. The primary benefits of using plant-derived medicine are that they are relatively safer than synthetic alternatives, offering profound therapeutic benefits and more affordable treatment. This research will provide scientific bases for the discovery of new therapeutic drugs from *Guiera senegalensis*. Amino acid analysis refers to the methodology used to determine the amino acid composition or content of proteins, peptides, and other pharmaceutical products. Amino acids such as lysine, arginine, histidine, glycine, phenylalanine, glutamic and aspartic has antimicrobial activity against some pathogens either in a pure state or conbined with other elements or compounds.(Fox, 2013). Metal (II) amino aids complex of glycine and phenylalanine produced antimicrobial activity against *Bacillus subtilis*, *Staphylococcus aureus*, Methicillin Resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris and Candida albicans.* (Temitayo *et al.*,2012). However, pure y-polyglutamic acids(y-PGA)from B. subtilis show antimicrobial activity against S. typhimuriun, S. aureaus and E.coli, especially,antimicrobial activity was higher against Gram-positive bacteria than against Gram-negative bacteria.Although a possible antimicrobial mechanism was reported by Inbaraj *et al.*,(2011).It has been well known that proliferation of bacteria is associated with its ability to adhere onto material surface(bacterial adhesion) through non-specific interactions such as electrostatic, hydrophobic, vander waals force and specific interactions between the bacterial cell membrane receptor and material surface. It has been well known that the proliferation of bacteria is associated with its ability to adhere onto material surfaces (bacterial adhesion) through non-specific interactions such as electrostatic, hydrophobic, vander Waals forces and specific interactions between the bacterial cell-membrane receptor and

material surface. (Inbaraj *et al.*,2011). That the growth of bacterial cells could be significantly reduced by materials that are hydrophilic and anionic and therefore the antimicrobial activity of the y-PGA may be due to the hydrophilic and anionic of the y-PGA.

1.2 Statement of Research Problem

Bacterial and fungal pathogens have evolved numerous defense mechanisms against antimicrobial agents, and resistance to old and newly produced drugs are on the rise (Hancock *et al*., 2012). Most of the drugs and even antibiotics are less active against the targeted organisms. We therefore witness the occurrence of antibiotic resistant organisms. In addition, majority of the orthodox drugs are both expensive and display numerous side effects on the users. As a result, managing patients especially in developing countries is rather expensive. Discovering and identifying new safe drugs without severe side effects has become an important goal of research in biomedical science. There is a challenge in the field of research to detect the gene that produced the functional protein which performs the antimicrobial activity. The plant *Guiera senegalensis* is used to treat numerous ailments, In view of this, the antimicrobial activity of the methanolic and aqueous extracts and amino acid profile of the partially purified protein of the *G. senegalensis* will be determined. This will increase the chances of finding new therapeutic agents and to get the best plant part for antimicrobial used.

1.3 Justification

Plants produce peptides and other secondary metabolites. Plants have the ability to synthesize a wide variety of chemical compounds that are used to perform important biological functions, and to defend against attack from predators such as insects, fungi and herbivorous mammals. At least 12,000 such compounds have been isolated so far; a number estimated to be less than 10 % of the total (Cederlund *et al.,* 2011). Antimicrobial peptides and secondary metabolites have been demonstrated to kill Gram negative and Gram positive bacteria, viruses, and fungi. (Al-Akeel *et al.,* 2014).In Nigeria over 90% of Nigerians in rural areas and about 40% in the urban areas depend partly or wholly on traditional medicine.(Ojua *et al.,* 2013). About 80% of the world's population relies on plant derived medicines for their primary health and 3.5 billion people in the developing world depend on the exploitation of medicinal plants and herbal products around them for their healthcare needs (Njimoh *et al.,* 2015)

1.4 Null Hypothesis

Guiera senegalensis leaf and root neither have amino acids nor antimicrobial activity.

1.5 Aim and Objectives

1.5.1 Aim of the Study

To evaluate the antimicrobial activity of methanolic and aqueous extracts and amino acid profile of the partially purified protein of *Guiera senegalensis*

1.5.2 Specific objectives

i. To determine the zone of inhibitions of *Guiera senegalensis* against the micro organisms.

ii. To determine the minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of the extracts.

iii. To determine the antimicrobial activity of the proteins.

iv. To evaluates the amino acids profile of the active partially purified proteins of the leaf and root of *G. senegalensis*.

v. To determine the molecular weight of the partially purified proteins using SDS-PAGE.

CHAPTER TWO

LITERATURE REVIEW

2.1 Traditional Medicine

Traditional medicine (TM) refers to health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illnesses or maintain well-being. Countries in Africa, Asia and Latin America use traditional medicine (TM) to help meet some of their primary health care needs. In Africa, up to 80 % of the population uses traditional medicine for primary health care (Thorsen *et al.,* 2015).

In industrialized countries, adaptations of traditional medicine are termed "Complementary" or "Alternative" medicine (CAM). Medicinal plants have been identified and used throughout human history. Plants have the ability to synthesize a wide variety of chemical compounds that are used to perform important biological functions, and to defend against attack from predators such as insect, fungi and herbivorous mammals At least 12,000 such compounds have been isolated so far; a number estimated to be less than 10 % of the total (Kalimuthu *et al.,* 2010). Chemical compounds in plants mediate their effects on the human body through processes identical to those already well understood for the chemical compounds in conventional drugs; thus herbal medicines do not differ greatly from conventional drugs in terms of how they work. Plants are one of the most important sources of medicine. Plant derived compounds (phytochemicals) have been attracting much interest as natural alternatives to synthetic compounds.

Extracts of plants were used for the treatment of various diseases and this forms the basis for all traditional systems of medicine (Kalimuthu *et al.,* 2010). The treatment and control of diseases by the use of available medicinal plants in a locality will continue to play significant roles in medical health care implementation in the developing countries (Ekundayo *et al.*, 2011). Various antifungal agents are currently available for the treatment and control of fungal infections and diseases. The use of these medicines as therapeutic agents however is limited. This is due to various challenges such as drug solubility, stability, adsorption and toxicity. In addition, some of these drugs are expensive and generally unavailable to citizens of developing countries, especially those residing in the rural areas (Sule *et al.,* 2011). The shortfalls in the use of chemotherapeutic agents as control agents in fungal diseases, further encourages the use of plants as a form of alternative medicine. Medicinal plants have been found as important contributors to the pharmaceutical, agriculture and food industries. With the onset of the synthetic era, pharmaceutical industries are producing a lot of synthetic drugs that help to alleviate the chronic diseases. With the passage of time many problems associated with frequent use of synthetic drugs become prominent like severe side effects and resistance of microbes against these drugs. On the other side synthetic drugs are expensive and a large population cannot afford these drugs. In recent times research on medicinal plants has been intensified all over the world.

Recently Phytochemical analysis, bioassays and the Identification of drug lead Compounds from Seven Bhutanese Medicinal Plants show that phenylpropanoids and furanocoumarins serve as antimicrobial and antimalarial constituents. (Phurpa *et al.*,2014).There is an emerging trend in research to support the biological activities of medicinal plants. Scientists have isolate phytochemicals from medicinal plants and many of them have been very active against many diseases.Such as tannins, flavonoids and other phenolic compounds (Maroyi, 2013).

2.2 Drug Discovery from Plants

Plants green leaves and other part are extremely beneficial to human health. Using green leaves as food, specific green leaves make excellent natural medicines (Sabandar, *et al.*, 2013). Leaves generally are very cleansing, healing, soothing and revitalizing as well as nourishing. For centuries, people have used plants for healing. Until recently, plants were important sources for the discovery of novel pharmacological active compounds, with many drugs being derived directly or indirectly from plants. Many modern drugs have their origin in the ethno-pharmacology (Al-Akeel *et al.*, 2014). Indeed, traditional medicine is a potential source of new drugs and as a source of cheap starting products for the synthesis of known drugs. These medicines initially took the form of crude drugs such as tinctures, teas, powders, and other herbal formulations. The specific plants to be used and the methods of application for particular ailments were passed down through oral history. Drug discovery from medicinal plants has evolved to include numerous fields of inquiry and various methods of analysis. Phytochemical studies have attracted the attention of plant scientists due to the development of new and sophisticated techniques. These techniques played a significant role in the search for additional resources of raw material for pharmaceutical industry (Shakeri et al., 2012)

The process typically begins with a botanist, ethno botanist, ethnopharmacologist, or plant ecologist who collects and identifies the plant(s) of interest. Collection may involve species with known biological activity for which active compound(s) have not been isolated (example, traditionally used herbal remedies) , Phytochemists (natural product chemists) prepare extracts from the plant materials, subject these extracts to biological screening in pharmacologically

relevant assays, and commence the process of isolation and characterization of the active compound(s) through bioassay-guided fractionation(Silverman and Holladay, 2014). The definition and practice of pharmacognosy have been evolving since the term was first introduced about 200 years ago as drug use from medicinal plants has progressed from the formulation of crude drugs to the isolation of active compounds in drug discovery. As practiced today, pharmacognosy involves the broad study of natural products from various sources including plants, bacteria, fungi, and marine organisms. Pharmacognosy includes both the study of botanical dietary supplements, including herbal remedies (Al-Akeel *et al.*, 2014).

Drug discovery from medicinal plants has played an important role in the treatment of diseases and, indeed, most new clinical applications of plant secondary metabolites and their derivatives over the last half century have been applied towards combating diseases.

2.3 The Plant *Guiera senegalensis*

2.3.1 Taxonomy of the plant

Kingdom:	Plantae
Phylum:	Angiosperms
Subphylum:	Eudicots
Class:	Rosids
Order:	Myrtales
Family:	Combretaceae
Genus:	*Guiera*
Species:	*senegalensis*

2.3.2 Common names

English:	Moshi medicine
Yoruba:	“oganwo”
Hausa:	“sabara”



Figure 2.1 Matured *Giuera Senegalensis* Plant Showing Flowers along Shika, 7 kilometers Giwa local Government Area, Kaduna State(Photo by Jiyil Markus).



Figure 2.2 Young *Guiera Senegalensis* Plant Showing Flowers along Shika,

7 kilometers Giwa local Government Area, Kaduna State(Photo by Jiyil Markus).

2.3.3 Botanical description

A shrub reaching 3 m high, of low rainfall areas and light dry soils occurring throughout the Sahel region from Mauritania to Nigeria, and across Africa to Sudan. The wood is whitish or tinged red, coarse grained, knotted and short, but very hard. It used in Sahel de Nioro of Mali for the framework of wells, bed-posts, etc. The shrub is commonly cut to fence farms.(Shettima *et al.*,2013)

2.3.4 Ecology and distribution of *Guiera senegalensis*

Guiera senegalensis occurs in shrub savanna tree and fallow land. from sea-level up to 1000 m altitude. it grows in areas with 200,400 and 800 mm annual rainfall. *Guiera senegalensis* occurs on all types of soil, sometime in area of temporarily flooded, and it does not tolerate heavy shading and is a very drought resistant plant.

2.3.5 Propagation and planting

Guiera senegalensis is propagated by seed, stem lying and root suckers. Seeds are sown in pots during the dry season and transplanted into the field when the rainy season is well established. Branch layering is done by simply burying young parts of stems during the rainy season until roots grow. Roots have been observed to grow within 2 weeks after layering.(Mamman *et al.*, 2013).

2.3.6 Disease and pests

Stem gall infection of *Guiera senegalensis* is very common and aphids are sometimes present, protected by ants, but other pests or diseases are rare (Shettima *et al.*,2013).

2.3.7 Uses of *Guiera senegalensis*

Guiera senegalensis is a well known medicinal plant and have been used in Burkina Faso as antioxidant and anti-inflammatory agent since ancient time (Sombie *et al.,* 2011). In Burkino Faso, *G. senegalensis* is used to treat fatigue, depression, anemia, nervous system disorders and bacterial diseases and also contributes in brain protection and inhibit hemolysis of erythrocytes (Nadembega *et al.*,2011).

The infusion of the whole plant, including the roots, is used to treat venereal diseases tuberculosis and stomach aches. The leaves are used as anti-dysenteric, febrifuge and diuretic. They are used as an infusion for the treatment of bronchus-pulmonary infections and diabetes, and as a gargle to treat mouth ulcers. They are also used as a decoction to treat intermittent fevers, colds and tuberculoses. Roots cut in small portions are used to clean teeth. In Senegal the water in which roots are boiled is used to heal diarrhoea, dysentery, pneumonias, bronchitis and colic. The powder made from the dry leaves mixed with food is used in West Africa to treat gastric ulcers and diarrhea (AY, and MA, 2012).

In Senegal, the decoction of leaves is used to treat cough and fever. The powder is applied to snakebites, in order to help to remove the poison and impede its entrance in the blood stream (Cotonou, 2012). The young fresh leaves are chewed to treat cough, bronchitis, fever, malaria, toothache and swollen gums. Inhaling the smoke - obtained from burning the dry leaves in a

pipe - through the nose is a traditional method used in the Gambia in order to treat colds. In Burkina Faso the dry leaves are ingested to treat fever and diarrhoea and are also used in the form of infusions and decoctions to treat dysentery, vomit, fever cholera and diarrhoea. The fresh crushed and macerated leaves are added to potash water to wash the head in order to treat headaches.

In Togo the leaves are used as a decoction through inhalation of the steam and baths to treat arthritis and rheumatism. When dried and reduced to powder, which is ingested with food, they are used to treat leprosy and asthma (.Dimobe *et al.,* 2012).. In western Africa, the ripe fruit is used in decocted form (on and through the mouth or inhalation of the steam) to treat nasal haemorrhages. The galls of *G. senegalensis* are used for the treatment of fowlpox and have antiseptic, antifungal activities. The root concoction is used to cure dysentery; diarrhoae and microbial infections. The ethanol extract of *Guiera senegalensis* possesses sufficient in vitro anticancer and antioxidant activities (Abubakar *et al.,* 2013).The plant has indolic alkaloids, tannins and flavonoids which provoke anti-inflammatory and anti- diarrhoeal effects.

2.4 Amino Acids, Peptides and Proteins

There are over 100 amino acids that exist in nature, human body requires 20 amino acids, called standard amino acids, for normal functioning. (Albanese, 2012). Protein structure is classified into four distinct levels: primary, secondary, tertiary, and quaternary. The primary structure is simply the actual amino acid sequence that makes up a protein prior to any folding. This interaction of the amino acids leads to formation of either an α-helix, β-sheetor random coil, which are synonymous with protein secondary structure. Tertiary structure results from the

interaction of multiple secondary structures as the protein folds and coils to attain a more compact three dimensional structure. Quaternary structure is composed of multiple tertiary structures formed into compact units called sub-units, which combine to form the overall protein.((Bonner and Varner, 2012).

2.4.1 Amino acid profile

Amino acid profile determines the amino acid composition or content of proteins, peptides, and other pharmaceutical preparations. Proteins and peptides are macromolecules consisting of covalently bonded amino acid residues organized as a linear polymer. The sequence of the amino acids in a protein or peptide determines the properties of the molecule (Ashkenazy *et al.,* 2010). Proteins are considered large molecules that commonly exist as folded structures with a specific conformation, while peptides are smaller and may consist of only a few amino acids. Amino acid profile can be used to quantify protein and peptides, to determine the identity of proteins or peptides based on their amino acid composition, to support protein and peptide structure analysis, to evaluate fragmentation strategies for peptide mapping, and to detect typical amino acids that might be present in a protein or peptide (Roberts *et al.,* 2012). It is necessary to hydrolyze a protein/peptide to its individual amino acid constituents before amino acid analysis.

2.5 Antimicrobial Proteins

Antimicrobial peptides (AMPs), are important part of the innate immune system, small molecules that may present antibacterial, antifungal, antiparasitic, and antiviral activity (Hancock and Diamond , 2000). Usually these molecules are composed of 10–50 amino-acid residues, and

arranged in different groups depending on the amino-acid composition, size, and conformation (Gallo, 2012). As well as having an immunomodulatory role, they also function in the protection against microbes. AMPs have promising therapeutic properties: they kill microbes rapidly, have broad activity-spectra and there are few reports of emerging bacterial resistance, and therefore much effort is focused on finding potential novel antibacterial drugs among AMPs. AMPs are widely distributed and have been found in organisms ranging from prokaryotes to plants, insects and mammals. They are generally considered as a part of the innate immune system and rapidly increase in concentration in the host upon challenge by pathogens. AMPs are expressed in many different cell types and tissues. AMPs are typically small molecules of amino acid (aa) residues in length and most are positively charged and amphipathic, containing both hydrophilic and lipophilic parts spatially separated. Some peptides are enriched for certain amino acids, such as the proline and arginine rich PR-39 or the histidine-rich histatins.

AMPs have been shown to have direct activity against a broad spectrum of microbes including a variety of Gram-positive and Gram-negative bacteria, fungi, viruses and protozoa (Jensen *et al.*, 2010).In recent years, it has become evident that several of these peptides also functions as modulators of both the innate and adaptive immune responses. It is likely that AMPs exert their action by both directly killing microbes as well as by stimulating the immune system. Plant antibacterial peptides are active against bacteria at low concentrations and have been identified in peripheral cell layers of seed, leaves and vegetative tissues, in accordance with their function as a primary defense of vulnerable tissues. Most peptides share some general characteristics such as positively charged residues and high cysteine content for the formation of disulphide bonds. (Mehra et al., 2012). However, some antibacterial peptides, such as the peptides isolated from

coconut water and the glycine-rich peptide from guava seeds, respectively, have acidic properties and no disulphide bridges (Tavares *et al.,* 2012). Most peptides have demonstrated activity against a broad range of different bacterial species and are therefore promising candidates for control of bacterial infections.

Many antibacterial peptide families have been isolated from plants. Pp-Thionin, for example, shows activity against Rhizobium meliloti. Moreover, Pp-AMP1 and Pp-AMP2 have potent activity against several phytopathogens. In addition, Circulins A-B show antibacterial effects against human pathogens such as *Staphylococcus aureus,Micrococcus luteus, Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris* and *Klebsiella oxytoca* at micromolar concentrations (Dowling *et al.,*2013). Furthermore, hevein-like proteins Ac-AMP1 and Ac-AMP1 cause growth inhibition of Bacillus megaterium.

2.5.1 C**haracteristic structures of antibacterial peptides**

Several studies of the structure of individual antibacterial peptides from plant sources have been performed, but only a few reports have made a comparison of their structural similarities and differences (Dowling *et al.,* 2013). Studies comparing the primary sequences and tertiary structures of antimicrobial peptides from plants show that 33 % of them present activity against bacteria, and around 59 % are formed by 30 to 50 amino acid residues (Dowling *et al.,* 2013). Moreover, it was observed that one key characteristic of antibacterial peptides is a high content of cysteine and/or glycine residues (Dowling *et al.,* 2013). The occurrence of disulphide bridges is also important for enhancing structural stability under diverse stress conditions (Dowling *et al.,* 2013). Additionally, it was observed that the percentage of cysteine residues is higher in peptides with known β-sheet structures (Guilhelmelli *et al.,*2013). This can be compared to an

antibacterial peptide belonging to the glycine-rich family and isolated from guava seeds (Tavares *et al.,* 2012). The structure of this peptide, inferred by molecular modelling studies, consists only of α-helices and lacks β-sheets. Analysis of the primary sequence revealed no cysteine residues, and thus the peptide is unable to form disulphide bonds (Dowling *et al.,* 2013). Therefore, evidence suggests that the presence of cysteine residues and β-sheet structures may go together, but this does not imply that these are relevant for antibacterial activity. Similar conclusions can be made concerning the presence of glycine residues. Glycine can provide flexibility to peptide structures, but nothing has been confirmed about its possible importance for antimicrobial activity (Dowling *et al.,* 2013).

However, there are implications that charged amino acids are relevant for activity against microorganisms. Around 17% of the amino acids in plant antimicrobial peptides are positively or negatively charged. Specifically, arginines and/or lysines comprise more than 70 % of all charged residues found in these peptides, while the remaining
30 % consists of the negatively charged aspartic acid and glutamic acid (Dowling *et al.,* 2013). As described further in this paper, charged residues seem to have an essential role in activity towards pathogenic bacteria.

Among all antibacterial peptides isolated and characterized from plant sources, only eight have been evaluated in terms of their tertiary structures. Three of these peptides belong to the cyclotide family, and four others are from the defensin group (Dowling *et al.,* 2013).
Puroindoline, similarly to indolicidin, is a family of peptides rich in tryptophan residues that also act by inhibiting DNA synthesis (Haney et al. 2013). Using radioactive precursors for DNA, RNA, and protein biosynthesis. Defensins have a typical three-dimensional structure composed

of a α-helix followed by 2-3 β-strands that are stabilised by 3-4 disulphide bridges (Dowling *et al.,* 2013). This structure can be observed in all members of this group; even among those with different functionality. Cyclotides are unique types of peptide in which the N- and C-termini interact to form a cyclic structure. They can be divided into the following two groups: the bracelets, the main feature of which is a three-dimensional structure composed of α-helices and strands (Méndez-Samperio, 2013). Nevertheless, although they present a cyclic conformation, cyclotide tertiary structure is very similar to that of peptides from the defensin family. To date, over 1,500 antimicrobial peptides have been listed in different databases.(Fjell *et al.,* 2007) These peptides are classified based on secondary structural features, such as cathelicidins (with a linear α-helical structure), defensins (with a β-strand structure), and bactenecins (with a loop structure) (Hof *et al.,* 2001). These peptides are also called cationic molecules because have a positive charge provided by arginine (Arg) and lysine (Lys) residues, and are small molecules (fewer than 100 amino acids in length). Currently, there are two main genetic categories for antimicrobial peptides in mammals: Cathelicidins and defensins.(Méndez-Samperio, 2013).

2.5.2 Mechanisms by which microorganisms exhibit resistance to antimicrobials

I. Drug inactivation or modification: for example, enzymatic deactivation of *penicillin* G in some *penicillin*-resistant bacteria through the production of β-lactamases. Most commonly, the protective enzymes produced by the bacterial cell will add an acetyl or phosphate group to a specific site on the antibiotic, which will reduce its ability to bind to the bacterial ribosome and disrupt protein synthesis (Guilhelmelli *et al.,* 2013).

II. Alteration of target site: for example, alteration of PBP—the binding target site of *penicillins*—in MRSA and other penicillin-resistant bacteria (Wieler *et al.,* 2011). Another protective mechanism found among bacterial species is ribosomal protection proteins. These proteins protect the bacterial cell from antibiotics that target the cell's ribosome to inhibit protein synthesis. The mechanism involves the binding of the ribosomal protection proteins to the ribosomes of the bacterial cell, which in turn changes its conformational shape. This allows the ribosome to continue synthesizing proteins essential to the cell while preventing antibiotics from binding to the ribosome to inhibit protein synthesis.

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

IV. Reduced drug accumulation: by decreasing drug permeability or increasing active efflux (pumping out) of the drugs across the cell surface these specialized pumps can be found within the cellular membrane of certain bacterial species and are used to pump antibiotics out of the cell before they are able to do any damage. These efflux pumps are often activated by a specific substrate associated with an antibiotic.(Brogden and Brogden, 2011).

2.5.3 Mechanism of action of antimicrobial agents

The main hypothesis for their mechanism of action involves the ability of AMPs to cause membrane collapse by interacting with lipid molecules on the bacterial cell surface. According to this hypothesis, the cationic peptides are attracted electrostatically to negatively charged molecules such as anionic phospholipids, lipopolysaccharides (LPS) (Gram-negative) and teichoic acid (Gram-positive), which are located asymmetrically in the membrane architecture (Wilson *et al.*,2011).

The positively charged residues can also interact with membrane lipids through specific receptors at the surface of the cell. Consequently, peptide binding to the membrane can activate several pathways that will cause cell death.

The following are the process by which antibiotics interfere with microbial cells:

A. Inhibition of Cell Wall Synthesis.

B. Inhibition of Nucleic Acid Synthesis.

C. Inhibition of Protein Synthesis.

D. Disruption of cell membrane.

E. Inhibition of metabolic activity.

2.5.3.1 *Inhibition of synthesis of cell wall peptidoglycan*

The bacterial cell, with the exception of a few is surrounded by a rigid wall that gives the microorganism shape and protection against damages from the environment (Prescott et al., 2013). Antimicrobial agents can prevent cell wall synthesis, simply by blocking the synthesis of peptidoglycan layer which covers the outer surface of the cytoplasmic membrane. Antimicrobial agents that disrupt the peptidoglycan structure and cause damages to the cell include *penicillins* and cephalosporins which are beta-lactams inhibiting the transpeptidases in bacterial cytoplasmic

membrane, thereby inhibiting the crosslinking of the peptidoglycan. Bacitracin and vancomycin are another cell wall inhibiting antibacterial agents. They interfere with the synthesis of linear strands of peptidoglycan.

2.5.3.2 *Inhibition of the nucleic acid synthesis*

A large number of agents interfere with purine and pyrimidine synthesis or with the interconversion or utilization of nucleotides. Other agents act as nucleotide analogs that are incorporated into polynucleotides. Antimicrobial agents may also bind to the enzyme gyrase to block DNA replication. ciprofloxacin and other quinolones as well as rifampin are particularly important in inhibiting nucleic acid synthesis. They inhibit bacteria DNA gyrase and thus interfere with DNA replication, transcription and other activities involving DNA. Rifampin blocks DNA synthesis by binding to and inhibiting the DNA-dependent RNA polymerase (Prescott et al., 2013). Mitomycin selectively inhibits DNA synthesis and griseofulvin interferes with both RNA and DNA metabolism and is effective against several bacterial (Prescott *et al*., 2013).

2.5.3.3 Inhibition of protein synthesis

A number of antibacterial agents act by inhibiting ribosome function. Bacterial ribosomes contain two subunits, the 50S and 30S subunits, binding to these sites cause protein chain termination and inhibit protein synthesis. Antibiotics that inhibit protein synthesis bind with the prokaryotic ribosome. Some drugs bind to the 30S ribosomal subunit while other attach to the 50S ribosomal subunit (Wieler *et al.,* 2011).

Aminoglycosides and tetracyclines bind with the 30S subunit thereby preventing the binding of the F-met-tRNA and aminoacyl-tRNA to the ribosome and this result in misreading. Chloramphenicol binds with 50S ribosomal subunit thereby inhibiting the activity of the peptidyl transferase responsible for peptide bond formation during protein synthesis (Prescott *et al*., 2013). The tetracyclines interfere with the attachment of the tRNA carrying the amino acids to the ribosome thereby preventing the addition of amino acids to the growing peptide chain.

2.5.3.4 Disruption of cell membrane

The cytoplasmic membrane controls the passage of nutrients and waste products into and out of the cell (Prescott *et al*., 2013). It also serves as site for respiratory and biosynthetic activities. Antimicrobial agents acting on the cell membrane include amphotericin B, nystatin which selectively inhibits microorganisms that possess ergosterol in their membrane, for example, Fungi. Nystatin therefore alters the membrane fluidity and perhaps produces pores in the membrane through which ions and small molecules are lost. Antimicrobial agents play role in distruption and destabilization of the cytoplasmic membrane

2.5.3.5 Inhibition of metabolic activities

Chemotherapeutic agents also inhibit microorganisms by interfering with some important metabolic processes. Typical examples of this group of agent are the sulphonamides that inhibit the biosynthesis of folic acid as structural analogue. These antimetabolites block metabolic pathways by completely inhibiting the use of metabolites by key enzymes. (Wieler *et al.*,

2011).Once bound to the membrane, they seem to make it permeable to ions and other cellular content, which causes great harm or even death to the cells. There are currently three different models to explain how antimicrobial peptides permeate bacterial cell membranes, as shown in Figure 2.3.

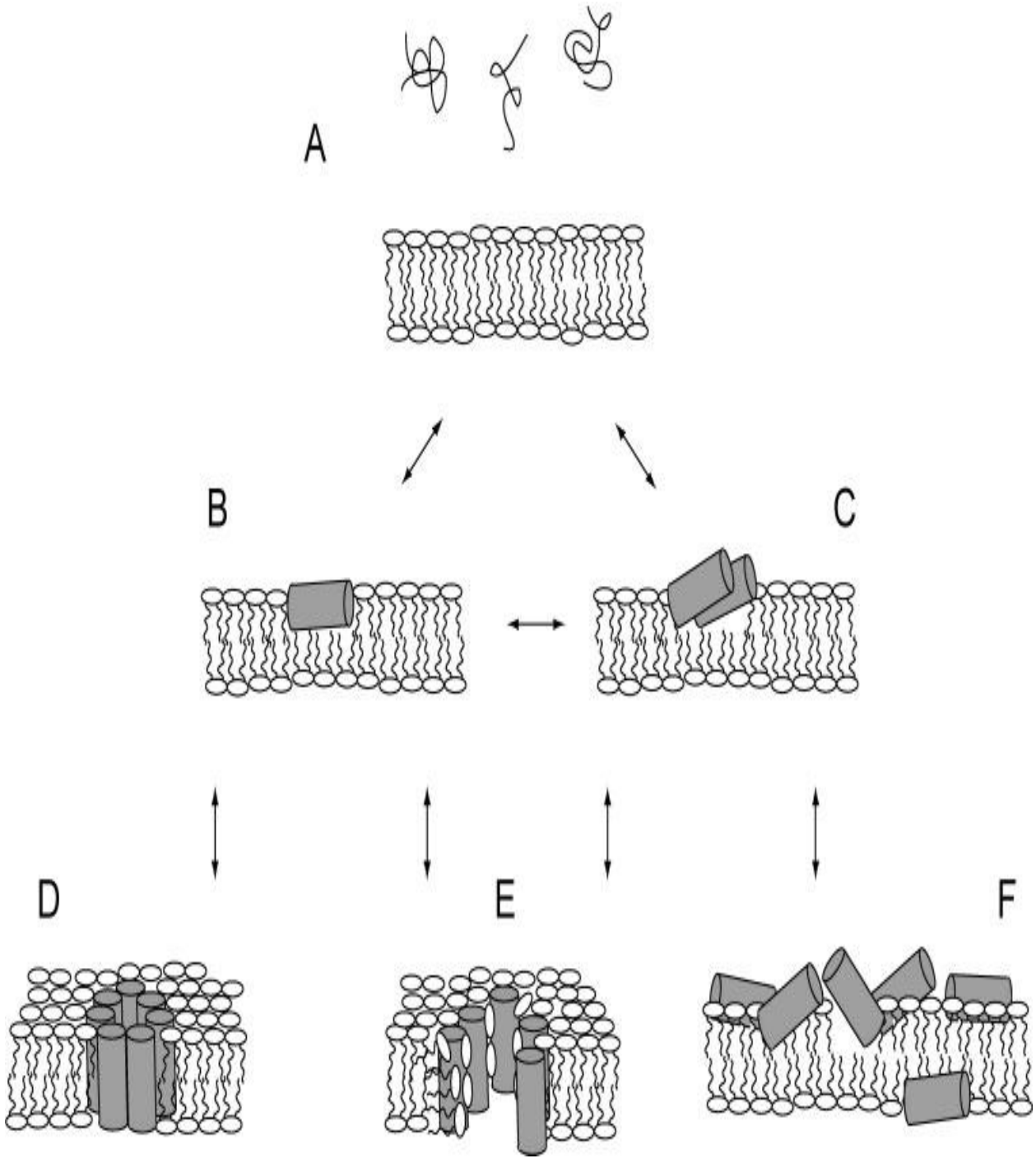


Figure 2.3 An overview of AMP membrane permeability models.(Bals, **2010**).

Figure 2.3 (A) shows the antimicrobial peptides in solution not yet bound to the lipid bilayer bacterial membrane. The antimicrobial peptide is bound to the membrane as a cylindrical monomer in (B), and multiple peptides have self-assembled and bound to the membrane in (C). The three models of membrane permeability are shown in (D), (E), and (F). In the first model, (D), the peptides form a pore across the membrane peptide molecules can be oriented perpendicularly, allowing their insertion into the lipid bilayer and the formation of transmembrane pores (Melo and Castanho, 2012). This is known as the "barrel-stave" model. The toroidal wormhole model shown in (E) appears very similar to (D), with a pore through the membrane, but closer examination shows negatively charged lipid head groups lining the pore that compensate for the positive charged peptides). The final model shown in (F) is known as the carpet model, where the membrane is simply disrupted by a high local concentration of peptides in a non structured manner(Bals, 2010).

2.6 Antimicrobial Agents

An antimicrobial or antibiotic is an agent that kills microorganism or inhibits their growth. Antimicrobial medicines can be grouped according to the microorganisms they act primarily against. For example, antibacterial are used against bacteria and antifungal are used against fungi. They can also be classed according to their function. Antimicrobials that kill microbes are called microbicidal; those that merely inhibit their growth are called microbiostatic. They kill a wide range of microbes and are valuable for cleaning inanimate surfaces to prevent the spread of illness. Examples aqueous extracts of yerba mate was active against Escherichia coli and

Staphylococcus aureus wish was due to the presences of some microbial agents like tannins, saponins and phenols (Burris *et al.*,2011).

2.6.1 Antimicrobial properties of medicinal plants

The use of crude extracts of plants parts and phytochemical, of known antimicrobial properties, can be of great significance in the therapeutic treatments of diseases. In recent years, a number of studies have been conducted in various countries to prove such efficiency. Many plants have been used because of their antimicrobial traits, which are due to the secondary metabolites synthesized by the plants. Leaves and flower of experimental plants have been used for treating many diseases in traditional medicines. The leaf of A. indica is commonly used against intestinal worms and some of poisonous bites such as Arizona coral snake and Gila monster bite and treat inflammation, cholera (Chinnaperumal *et al.,* 2012) and the essential oil possess anti-microbial activity against *Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus aureus , E. coli, Bacillus subtilis and Salmonella typhimurium*.

A study was reported on the aqueous extract from the artichoke (*Cynara scolymus*) and the ethanol extracts (80%) from both artichoke and "macela" (*Achyrocline satureioides*) inhibited the growth of *Bacillus cereus*, *B. subtilis*, *Pseudomonas aeruginosa* and *S. aureus* (Asolini *et al*., 2006). In Argentina, terpene compounds (eugenol, geraniol, thymol and carvacrol) derived from essential oils of native plants showed inhibitory effects on MRSA (Chinnaperumal *et al.*,2012).

2.6.2 Antibacterial

Antibacterial is anything that destroys bacteria or suppresses their growth or their ability to reproduce. Antibacterials are used to treat bacterial infections. The toxicity to humans and other animals from antibacterial is generally considered low. The discovery, development and clinical use of antibacterial during the 20th century have substantially reduced mortality from bacterial infections (Taylor *et al.,* 2013).

. Antibacterial are among the most commonly used drugs. For example 30% or more patients admitted to hospital are treated with one or more courses of antibacterial. Antibacterial may be divided into two groups according to their speed of action and residue production: The first group contains those that act rapidly to destroy bacteria, but quickly disappear (by evaporation or breakdown) and leave no active residue behind (referred to as *non-residue-producing*). Examples of this type are the cobalt, chlorine, peroxides, and aldehydes (Chang *et al.,* 2010).The second group consists mostly of newer compounds that leave long-acting residues on the surface to be disinfected and thus have a prolonged action (referred to as *residue-producing).*

2.6.3 Antifungal

A medication that limits or prevents the growth of yeasts and other fungal organisms, Antifungal are used to kill or prevent further growth of fungi. In medicine, they are used as a treatment for infections such as athlete's foot ringworm and thrush and work by exploiting differences between mammalian and fungal cells. They kill off the fungal organism without dangerous effects on the host (Silva *et al.,* 2012).

2.6.4 Antiviral

Antiviral drugs are class of medication used specifically for treating viral infections. Like antibiotics, specific antiviral are used for specific viruses. They are relatively harmless to the host and therefore can be used to treat infections. They should be distinguished from viricides which actively deactivate virus particles outside the body. Many of the antiviral drugs available are designed to treat infections by retroviruses, mostly HIV (De Clercq *et al.*, 2013).

. Important antiretroviral drugs include the class of protease inhibitors. Herpes viruses best known for causing cold sores and genital herpes are usually treated with the nucleoside analogue acyclovir. Viral hepatitis (A-E) is caused by five unrelated hepatotropic viruses and is also commonly treated with antiviral drugs depending on the type of infection (Lok *et al.*, 2012). Many antiviral agents have been isolated from plant sources and have been partly or completely characterised.

Table 2.1. Plants Possessing Antimicrobial Activity

Common Name	Scientic Name	Compound	Class	Activity
Allspice	*Pimento dioica*	Eugenol	Essential oil	Bacteria , fungi and viruses
Aloe	*Aloe vera*	Latex	Complex mixture	Corynebacterium,salmonella,
Apple	*Malus sylvestris*	Phloretin	Flavonoid derivative	Streptococcus, S. aureus
Cashew	*Anacardium pulsatilla*	Salicylic acids	Polyphenols	Bacteria, fungi and virus
Chili peppers,	*Capsicum annuum*	Capsaicin	Terpenoid	P. acnes
Garlic	*Allium sativum*	Allicin, ajoene	Sulfoxide	Bacteria, fungi
Grapefruit peel	*Citrus paradise*	Asiatocoside	Terpenoid	Bacteria
Green tea	*Camellia sinensis*	Catechin	Flavonoid	Bacteria , Fungi and viruses
Lemon balm	*Melissa officinalis*	Tannins	Polyphenols	Fungi
Olive oil	*Olea europaea*	Hexanal	Aldehyde	Bacteria , Fungi and viruses
Onion	*Allium cepa*	Allicin	Sulfoxide	Viruses, Candida

Source: adapted from Cowan (Cowan, 1999)

2.7 Overview of Test Organisms

2.7.1 *Staphylococcus aureus*

Bacteria of the genus *Staphylococcus* are Gram-positive cocci that are microscopically observed as individual organisms, in pairs and in irregular, grapelike clusters. *Staphylococci* are non-motile, non-spore forming and catalase-positive bacteria. The cell wall contains peptidoglycan and teichoic acid. The organisms are resistant to temperatures as high as 50^{o}C, to high salt concentrations, and to drying. Colonies are usually large (6-8mm in diameter), smooth and translucent. The colonies of most strains are pigmented, varying from cream-yellow to orange (Hanahoe *et al.,* 2009).The ability to clot plasma continues to be the most widely used and generally accepted criterion for the identification of *Staphylococcus aureus*. One such factor, bound coagulase, also known as clumping factor, reacts with fibrinogen to cause organisms to aggregate. Another extracellular staphylo-coagulase reacts with prothrombin to form staphylo thrombin, which can convert fibrinogen to fibrin. Approximately 97 % of human *S. aureus* isolates possesses both of these forms of coagulase (Hanahoe *et al.,* 2009).

The postulated sequence of events that leads to infection is initiated with carriage of the organism. The organism is then disseminated through hand carriage to body sites where infection may occur (either through over breaks in dermal surfaces, such as vascular catheterization or operative incisions, or through less evidence breakdown in barrier function, such as eczema or sharing-associated microtrauma (Rivero-Pérez, *et al.,* 2012).The hallmark of staphylococcal infection is the abscess which consists of a fibrin wall surrounded by inflamed tissues enclosing a central core of pus containing organisms and leukocytes. From this focus of infection, the

organisms may be disseminated hematogenously, even from the smallest abscess. The ability to elaborate proteolytic enzymes facilitates this process. This may result in pneumonia, bone and joint infection and infection of the heart valves. (Ostrowsky *et al.,* 2013).

2.7.2 *Bacillus subtilis.*

Bacillus subtilis, known also as the hay *Bacillus* or grass *Bacillus*, is a Gram-positive, catalase positive bacterium, found in soil, the gastrointestinal tract of ruminants and humans(Cairns, 2014). A member of the genus *Bacillus*, *B. subtilis* is rod-shaped, and has the ability to form a tough, protective endospore, allowing the organism to tolerate extreme environmental conditions.*Bacillus subtilis* can also be found in the human body, mostly on the skin or in the intestinal tract.*Bacillus subtilis* also produces a toxin called subtilisin. Subtilisin can cause allergic reaction if there is repeated exposure in high concentrations.

2.7.3 *Esherichia coli*

E. coli belongs to the large, heterogenous group of Gram negative rods referred to as enterobacteriaceae whose natural habitat is the intestinal tract of humans and animals. The Enterobacteriaceae are facultative anaerobes or aerobes, non-sporing, non-motile or motile, ferment glucose and produce a variety of toxins and other virulent factors. They also possess a complex antigenic structure (Brooks *et al.,* 2007). *E. coli* is the commonest cause of urinary tract infection (UTI) and accounts for approximately 90 % of first UTIs in young women. UTI can result in bacteremia with clinical signs of sepsis (Gould *et al.,* 2013). *E. coli* may reach the bloodstream and cause sepsis when the normal host defenses are inadequate. Newborns may be highly susceptible because they lack IgM antibodies (Brooks *et al.,* 2007). *E. coli* also cause diarrheal disease. These *E. coli* are classified by the characteristics of their virulence properties

and each group causes disease by a different mechanism. The toxins are often plasmid-or phagemediated.

2.7.4 *Salmonella typhimurium*

Salmonellae are Gram negative rods, belonging to the family enterobacteriaceae. They are found in virtually all animals, birds (including poultry), reptiles, rodents, domestic animals and humans ((Brooks *et al.*, 2007). They are non-sporing and with the exception of *S. typhi*, non-capsulate. They are facultative anaerobic. They grow between 15-45^{0}C with an optimum temperature of 37^{0}C Salmonellae grow readily on a wide range of simple media, but they never almost ferment lactose or sucrose. They are distinguished from other members of the family by their biochemical characteristics and antigenic structure. They usually produce hydrogen sulphide (H_2S). They survive freezing in water for long periods. They are resistant to certain chemicals (example, brilliant green, sodium deoxycholate) that inhibit other enteric bacteria

Salmonella spp cause many types of infections, from mild self-limiting to life threatening systemic disease such as typhoid fever. Infections with *S.enterica* serotypes typhi and paratyphi are mainly encountered (Harris, 2014).

Salmonellae penetrate the intestinal epithelial lining, multiply and produce three main types of disease in humans. Enteric fever (typhoid and paratyphoid fever): Produced by only a few of the *salmonellae,* of which *S. typhimurium*(typhoid fever) is the most important. The ingested *salmonellae* reach the small intestine, from which they enter the bloodstream. They are carried by the blood to many organs, including the intestine. After an incubation period of 10 –14 days, fever, malaise, headache, constipation, enlargement of the spleen, nausea, mental confusion,

intestinal hemorrhage, necrosis of the tissue, focal necrosis of the liver, inflammation of the gall bladder, Rose spots (rash) and normal or low white blood cell count (Brooks *et al.*, 2007).

2.7.5 *Candida albicans*

C. albicans is the commonest cause of candidiasis. The yeast is a common commensal of the skin, mucous membranes and gastrointestinal tract. Most *Candida* infections are opportunistic, occurring in debilitated persons. *Candidiasis* is also associated with prolonged broad-spectrum antibiotic therapy (Brooks *et al.*, 2007).

Many different clinical forms of candidiasis are known, involving primarily the mucosal surfaces (thrush), gastrointestinal and deep-seated infections such as candidaemia or meningitis. *Candida vaginitis* is a common infection during pregnancy. *Candida* infections of the mouth and oesophagus are common in those with HIV infection ((Brooks *et al.*, 2007). *Candida yeasts* are small, oval, measuring 2 – 4m in diameter. In stained smears, the yeasts can often be seen attached to *pseudohyphae*. Both yeasts and *pseudohyphaes* are Gram positive. The yeast cells can also be detected in unstained wet preparations. *C. albicans* grows on Sabouraud agar and most routinely used bacteriological media. Creamcoloured pasty colonies usually appear after 24 – 48hours incubation at35 – 37^{o}C. The colonies have a distinctive yeast smell and the budding yeast cells can be easily seen by direct microscopy in stained or unstained preparations (Mayer *et al.*, 2013).

CHAPTER THREE

MATERIAL AND METHODS

3.1 Materials

3.1.1 Sample Collection

Fresh leaves and roots of *Guiera senegalensis* were collected from Shika, Giwa Local Government Area of Kaduna State, Nigeria. They were authenticated at the Haberium Department of Biological Sciences, Ahmadu Bello University Zaria Kaduna State.

Voucher Number of *Guiera senegalensis* :1823.

3.2 Method

3.2.1 Preparation of Extracts

The fresh leaf and root of young *G. senegalensis* were washed with distilled water and dried at room temperature for one month . They were pulverized using a mechanical grinder. The powdered plant material 50 g was extracted in methanol (300 ml) and cold water (300ml) as detailed below according to method of Atawodi *et al*.,2004.

3.2.2 Methanol extraction of dried leaves of *G. senegalensis.*

Exactly 50 g of dried leaf powdered was extracted in a soxhlet apparatus with 300 ml methanol. The solution of the methanol extract was gently evaporated to dryness in a water bath at 40 ^{0}C in fume cupboard. The resultant crude extract was transferred into airtight sample bottles and kept at 4 ^{0}C until when required. This was repeated for dried roots materials.

3.2.3 Cold water extraction of dried leaf *G. senegalensis*

Exactly 50 g of dried leaf powdered was soaked in 300 ml distilled water in a 500 ml sterile conical flask with a constant stirring using magnetic stirrer. The mixture was allowed to stand at room temperature for 48 hours, after which it was filtered using No. 1 Whatman filter paper. The filtrate was gently evaporated to dryness in a water bath at 40 ^{o}C and stored in a refrigerator at 4 ^{o}C until when required. The same was repeated for dried roots materials.

3.2.4 Preparation of stock solution of extract

The stock solutions of the eight extracts, methanol extracts of matured leaf, young leaf, matured root, young root and aqueous extracts of matured leaf, young leaf, matured root and young root were prepared by dissolving 1.0 g of each extract in 10 ml of sterile distilled water to give a concentration of 100 mg/ml. The stock solutions were reconstituted to graded concentrations of 50 mg/ml, 25 mg/ml and 12.5 mg/ml using two-fold dilution. They were well labelled and stored at 4 ^{0}C until when required.

3.2.5 Preparation of test organisms

The stock bacterial and fungal isolates used were obtained from Ahmadu Bello University Teaching Hospita Zaria, Kaduna State. Fresh pure plates of the test organisms were made from the isolate cultures obtained on agar slants. The isolates were sub-cultured on selective and differential solid media and re-identified using colony morphology, gram reaction, motility test, haemolytic activity and biochemical tests namely catalase, bile solubility, litmus milk, citrate, oxidase and fermentation of sugars- mannitol, lactose and sorbitol (Cheesbrough, 2002). With the aid of sterile wire loop, colonies of fresh cultures of the different bacterial isolates were

picked and suspended in 5 ml nutrient broth in a well-labelled sterile 10ml bijou bottles. They were incubated at 37 ^{0}C for 24 hours.

3.2.6 Determination of preliminary antimicrobial activity of extracts

The antimicrobial activity of the extracts were determined using agar well diffusion test and broth dilution technique (Adeniyi *et al.*, 2013).The antimicrobial activity of the plant extracts was tested on four standard bacteria species and fungus namely; *Bacillus subtilis* , *Staphylococcus aureus* ,*Eschericia coli* , *Salmonella typhimurium* and *Candida albican* in the Microbiology Laboratory, Faculty of Sciences Ahmadu Bello University Zaria. These were standard laboratory cultures whose susceptibility on commonly used antibiotics was already established, *Staphylococcus aureus* and *Bacillus subtilis* represented gram positive bacteria while *Eschericia coli* and *Salmonella typhimurium* represented gram negative bacteria.

3.2.7 Agar well diffusion assay

The agar well diffusion technique as modified by Ali *et al.*, (2014) was the standard method used to determine the antibacterial activity of the bioactive compounds. Briefly in the method, the media of Mueller hinton agar (Becton Dicknson M.D USA) and while the potatoes dextrose agar was prepared and treated according to manufacturer's guidelines, where 38g of mueller hinton agar was mixed with one litre of distilled water and enclosed in a container and autoclaved at 121 ^{o}C for 15 minutes. The media was later dispensed into 90mm sterile agar plates (Oxoid, UK) and left to set for bacterial assay while19 g of potatoes dextrose agar was mixed with one litre of distilled water and autoclaved at 121 ^{o}C for 15 minutes for fungus assay . The agar plates were incubated for 24 hours at 37 ^{o}C to confirm their sterility. Absence of growth after 24 hours

showed that the plates were sterile. The Sterile agar plates were inoculated with the test culture by surface spreading using sterile wire loops and each organism evenly spread on the entire surface of the plate to obtain uniformity of the inoculum. The culture plate then had at most 4 wells of 6 mm diameter and 5mm depth made into it using a sterile agar glass borer. Ciprofloxacin was used as a positive control for bacteria and ketoconazole for fungus. Approximately 0.2 ml of the bioactive test compound of concentration 100 mg/ml,50 mg/ml, 25 mg/ml and 12.5 mg/ml was suspended in the wells and thereafter inoculated plates/culture were incubated for 24 hours at 37 ^{0}C. The plates/cultures were examined for the presence of inhibition zones around each well.

Antimicrobial activity was determined from the zone of inhibition around the wells. Single readings were carried out. Non-active compounds did not show any inhibition zone. The zones of inhibition were measured using a ruler and a pair of divider and results were reported in millimetres (mm). All zone diameters were considered important since the extracts from the plants were still crude. A zone size interpretive chart was then drawn to show the different plant extracts and their corresponding inhibition zone diameter to the nearest millimetre.

3.2.8 Determination of minimum inhibitory concentration (MIC)

The MIC was evaluated on plant extracts which showed activity on any bacteria organism. The method used was the tube dilution method (Adesokan *et al*., 2007).The plant extracts were serially diluted from the solutions of 50 mg/ml to obtained varying concentration .The concenration were; 25 mg/ml, 12.5 mg/ml, 6.25mg/ml, and 3.125 mg/ml.doubling dilutions of the extract were incorporated in Muller Hinton broth (Oxoid, UK) and then inoculated with 0.1ml each of standardized suspension of the test organisms into the various test tube containing varying concentrations and another set of test tubes containing only Mueller Hinton broth were

used as negative control, another test tube containing Mueller Hinton broth and test organisms were used as positive control.All the test tubes and controls were then incubated at 37 ^{O}C for 24hrs. After incubation period, the presence or absence of growth on each tube was rated using the following scale: - = no growth, + = scanty growth, ++ =moderate growth, +++ =heavy growth. a loop full from each tube was further sub cultured on nutrient agar to comfirmed weather the bacterial growth was inhibited. Growth of bacteria on solid media indicated that particular concentration of the extract was unable to inhibit the bacteria. The lowest concentration of extract showing no growth indicated the amount of extract in grams per millilitre to which the organism is susceptible. This was the minimum inhibitory concentration (MIC).

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3.2.9 Determination of minimum bactericidal concentration (MBC)

The MBC was determined by collecting 1ml of broth culture from the tubes used for the MIC determination and subculturing into fresh solid nutrient agar plates. The plates were incubated at 37 ^{0}C for 24 h. The least concentration that did not show any growth after incubation was regarded as the MBC (Adesokan *et al*., 2007).

3.2.10 Partial purification of antimicrobial proteins/peptides from leaf and root of *G. senegalensis*

Antimicrobial proteins and peptides was analysed using method of Bibiana and Selvamani, 2014) with slight modification in buffer concentration. The fresh leaves and roots samples of 50 g each were homogenized using 0.1 M phosphate phosphates buffer, pH 7.4 and then filtered. The crude sample was centrifuged at 10,000 rpm for 30mins. The crude extracts were saturated with 80 % ammonium sulfate. The saturated extract was subjected for dialysis. After dialysis

these samples were subjected to spectrophotometric analysis to determine the concentration of the protein. The supernatant was subjected to gel filtration chromatography using Sephadax G-15 Approximately 40 fractions (3.0 ml each) were collected at the flow rate of 1ml/21seconds with potassium phosphate as eluting buffer and absorbance (OD) was measured at 280 nm.

3.2.11 Antimicrobial assay of crude and partial purified proteins extracts

Purified protein fractions obtained after gel filtration chromatography were also tested for their antimicrobial activity by agar well diffusion method. The fractions showing maximum antimicrobial activity were then taken for SDS-PAGE to determine the molecular weight of the protein. Antimicrobial activity was expressed in arbitrary units (AU/ml). One AU was defined as the reciprocal of the highest level of dilution resulting in a clear zone of growth inhibition (Teixeira *et al.*,2013)

3.2.12 SDS-PAGE of most active partial purified proteins

One dimension SDS-PAGE was carried out following modified method of Laemmli, 1970. SDS-PAGE was run on vertical slab gel system. Proteins were electrophorised on 12 % separating gel (0.75 mm thickness) overlaid with 5 % stacking gel. A 10 % (w/v) stocksolution of precipitated protein in deionized water was run in SDS-PAGE (Boobathy *et al*., 2009).

3.2.13 Determination of amino acid profile

The Amino Acid profile of the sample was determined using methods described by Benitez (1989). The leaves and roots samples were dried to constant weight, defatted, hydrolyzed, evaporated in a rotary evaporator and loaded into the Technicon sequential Multi-Sample Amino Acid Analyzer (TSM) as shown below according to the method of AOAC, 2006.

3.2.14 Nitrogen determination

Amount (200 mg) of sample was weighed, wrapped in whatman filter paper (No.1) and put in the Kjeldal digestion flask. Concentrated sulphuric acid (10 ml) was added. Catalyst mixture (0.5 g) containing sodium sulphate (Na_2SO_4), copper sulphate ($CuSO_4$) and selenium oxide (SeO_2) in the ratio of 10:5:1 was added into the flask to facilitate digestion. Four pieces of anti-bumping granules were added. The flask was then put in Kjeldal digestion apparatus for 3 hours until the liquid turned light green. The digested sample was cooled and diluted with distilled water to 100 ml in standard volumetric flask. Aliquot (10 m1) of the diluted solution with 10 ml of 45 % sodium hydroxide was put into the Markham distillation apparatus and distilled into 10 ml of 2 % boric acid containing 4 drops of bromocresol green/methyl red indicator until about 70 ml of distillate was collected. The distillate was then titrated with standardized 0.01 N hydrochloric acid to grey coloured.

$$PercentageNitrogen = \frac{(a-b) \times 0.01 \times 14 \times V \times 100}{W \times C}$$

Where:

a. = Titre value of the digested sample

b. = Titre value of blank sample

v. = Volume after dilution (100ml)

W. = Weight of dried sample (mg)

C. = Aliquot of the sample used (10ml)

14. = Nitrogen constant in mg.

Percentage protein = % of Nitrogen × 6.25.

3.2.15 Defatting sample:

The sample was defatted using chloroform/methanol mixture of ratio 2:1. 4 g of the sample was put in extraction thimble and extracted for 15 hours in soxhlet extraction apparatus (AOAC, 2006).

3.2.16 Hydrolysis of the sample

A weight of 2.0 g of defeated sample was weighed into glass ampoule. 7 ml of 6 N HCL was added and oxygen was expelled by passing nitrogen into the ampoule (this is to avoid possible oxidation of some amino acids during hydrolysis (example methionine and cysteine). The glass ampoule was then sealed with Bunsen burner flame and put in an oven preset at 105 ^{0}C ± 5 ^{0}C for 22 hours. The ampoule was allowed to cool before open at the tip and the content was filtered to remove the humans. It should be noted that tryptophan is destroyed by 6 N HCL during hydrolysis. The filtrate was then evaporated to dryness at 40 ^{0}C under vacuum in a rotary evaporator. The residue was dissolved with 5ml to acetate buffer (pH 2.0) and stored in plastic specimen bottles, which were kept in the freezer.

i. Loading of the Hydrolysate into TSM analyzer

Between 5 to 10 micro litres was injected into the cartridge of the analyzer. The TSM analyzer is designed to separate and analyze free acidic, neutral and basic amino acids of the hydrolysate. The period of an Analysis lasted for 76 minutes.

iiMethod of Calculating Amino Acid Values from the Chromatogram Peaks

An integrator attached to the Analyzer calculates the peak area proportional to the concentration of each of the amino acids.

Alternatively, the net height of each peak produced by the chart recorder of TSM was measured. The half-height of the peak on the chart was found and width of the peak on the half height was accurately measured and recorded. Approximately area of each peak was then obtained by multiplying the height with the width at half-height.

The norcleucine equivalent (NE) for each amino acid in the standard mixture was calculated using the formula.

$$NE = \frac{Area\ of\ Norleucine\ \ Peak}{Area\ \ of each\ amino\ acid}$$

A constant S (standard amino acid) was calculated for each amino acid in the standard mixture:

Where $S_{std} = NE_{std}$ x Molecular Weight x μAA_{std}

Finally, the amount of each amino acid present in the sample was calculated in g/16 g N or g/100 g protein using the following formula:

Concentration (g/100g protein) = NH x W at NH/2 x S_{std} x C

Where $C = \left(\frac{Dilution \times 16}{Sampleweight(g) \times 10 \times Volumeloaded}\right) \div (NH \times W(nleu))$

Where: NH = Net height

W = Width at half height

nleu = Norleucine

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4.0 RESULTS

4.1 Antimicrobial screening of *Guiera senegalensis*

Table 4.1 shows the results of the antimicrobial screening of different concentrations of the extracts on the following isolates: *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Salmonella typhimurium* and *candida albicans*for methanol and cold water extracts of the young and matured leaves and roots of *G. Senegalensis*. The results showed that increased in concentration of extracts increased the zone of growth inhibition of the micro organism. A broad spectrum antibiotic (ciprofloxacin 100 mg/ml) and antifungal (Ketoconazole100 mg/ml) were used as standard drugs against the cultured organism. The outcome of the antibiotic result indicated that, *Bacillus subtilis* showed higher zone of inhibition (45.33 ± 1.53 mm) followed by *salmonella typhimurium* (36.33 ±2.08 mm), *Staphylococcus aureus* (35.00 ±1.00 mm), *Escherichia coli* (22.33 ±1.53 mm) and the antifungal zone of inhibition against *Candida albicans* was 40.00 ±1.00 mm at a concentration of 100 mg/ml. The zone of the inhibitions were used as a standard to compare with the zone of inhibition of extracts. All the extracts were susceptible to all the bacteria isolates but unsusceptible to fungus. The tables below shows the zone of inhibition of all the extracts at different concentrations (100 mg/ml, 50 mg/ml, 25 mg/ml and 12.5 mg/ml).

Table 4.1 Antimicrobial screening of different concentrations of methanol extracts of matured leaf of *Guiera senegalensis*.

Zone of inhibition of micro organisms (mm)

Concentration of extract/Standard drugs(mg/ml)	*Bacillus subtilis*	*Staphylococcus aureus*	*Escharichia coli*	*Salmonella typhimurium*	*Candida albican*
100	17.33 ± 0.58^{b}	17.67 ± 0.58^{b}	18.00 ± 1.00^{b}	16.67 ± 1.15^{b}	NI
50	15.67 ± 0.58^{bc}	16.33 ± 0.58^{b}	15.33 ± 0.58^{bc}	15.33 ± 0.58^{b}	NI
25	15.33 ± 0.58^{bc}	$14.00 \pm 1.00c$	12.67 ± 0.58^{bc}	11.33 ± 1.00^{c}	NI
12.5	12.00 ± 1.00^{bc}	12.33 ± 0.58^{c}	12.00 ± 1.00^{bc}	NI	NI
Cipr 100	45.33 ± 1.53^{a}	35.00 ± 1.00^{a}	22.33 ± 1.53^{a}	36.33 ± 2.08^{a}	NI
Keto.100					40.00 ± 1.00^{a}

(^{a,b,c}) = Means in the same column with different superscripts letter indicates statistically significant differences ($P < 0.05$). Values are mean± standard deviation of triplicate. NI = No inhibition.Diameters of zones inhibition ≥ 10mm exhibited by plant extract were considered active(Usman *et al*.,2008).Standard drugs: Cipr = Ciprofloxacin(Antibacterial drug) , keto=Ketokonazole (Antifugal drug)

Table 4.2 shows zone of inhibitions for methanol extract of young leaves. All the organism were sensitive at all concentration except *Salmonella typhimurium* and *candida albicans. Stahylococcus aureus* showed higher zone of inhibition in all the concentrations of the extract with maximum zone of inhibition(42.00 ± 1.00 mm) at 100 mg/ml followed by (41.00 ± 1.00 mm) at 50mg/ml while the minimum zone (11.67 ± 0.58 mm) was observed in *Bacillus subtilis* at 12.5 mg/ml. The zones of inhibition at 100 mg/ml and 50 mg/ml concentrations of the extract were higher than the zone of inhibition (35.00 ± 1.00 mm) of standard drug(ciprofloxacin) at 100mg/ml against *Staphylococcus aureus*. The zones of inhibitions of the extract and that of the antibiotics were statistically significant ($P \leq 0.05$).

Table 4.2 Antimicrobial screening of different concentrations of methanol extracts of young leaves of *Guiera senegalensis*

Zones of inhibition (mm)

Concentration of extract/ Standarddrugs (mg/ml)	*Bacillus Subtilis*	*Staphylococcus Aureus*	*Escharichia Coli*	*Salmonella typhimurium*	*Candida albican*
100	18.33 ± 0.58^{b}	42.00 ± 1.00^{b}	21.67 ± 1.53^{b}	NI	NI
50	16.00 ± 0.58^{b}	41.00 ± 1.00^{b}	12.33 ± 0.58^{b}	NI	NI
25	15.33 ± 0.58^{C}	35.00 ± 1.00^{C}	12.33 ± 0.58^{C}	NI	NI
12.5	11.67 ± 0.58^{C}	15.33 ± 1.00^{C}	12.00 ± 1.00^{C}	NI	NI
Cipr 100	45.33 ± 1.53^{a}	35.00 ± 1.00^{a}	22.33 ± 1.53^{a}	36.33 ± 2.08^{a}	NI
Keto.100					40.00 ± 1.00^{a}

(^{a,b,c}) = Means in the same column with different superscripts letter indicates statistically significant differences ($P < 0.05$). Values are mean± standard deviation of triplicate. NI = No inhibition.Diameters of zones inhibition ≥ 10mm exhibited by plant extract were considered active(Usman *et al*.,2008).Standard drugs: Cipr = Ciprofloxacin(Antibacterial drug) , keto=Ketokonazole (Antifugal drug)

Table 4.3 shows zone of inhibitions of methanol extract of matured roots. All extract concentrations were sensitive to *Staphylococcus aureus,* and *Salmonella typhimurium* but insensitive to *Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Candida albicans.*

At 100 mg/ml and 50 mg/ml there was no significant differences on the zones of inhibitions between *Bacillus subtilis* (17.00 ± 1.00 and 16.00 ± 1.00) and *Staphylococcus aureus* (18.33 ± 0.58mm and 16.33 ± 1.00) respectively while the minimum zone of inhibition (11.33 ± 0.58) was observed in *Bacillus subtilis* at 12.5mg/ml.this indicates that *Bacillus subilis* and *Staphylococcus aureus* were more susceptible to the extract.

Table 4. 3 Antimicrobial screening of different concentrations of methanol extracts of matured root of *Guiera senegalensis.*

	Zones of inhibition of micro organisms (mm)				
Concentration of extract/Standard drugs (mg/ml)	*Bacillus subtilis*	*Staphylococcus Aureus*	*Escharichia coli*	*Salmonella typhimurium*	*Candida albican*
100	18.67 ± 1.15^{b}	19.33 ± 1.53^{b}	NI	NI	NI
50	16.67 ± 0.58^{b}	15.33 ± 1.15^{C}	NI	NI	NI
25	15.33 ± 0.58^{bc}	12.67 ± 1.53^{bc}	NI	NI	NI
12.5	12.00 ± 1.00^{bc}	NI	NI	NI	NI
Cipr 100	45.33 ± 1.53^{a}	35.00 ± 1.00^{a}	22.33 ± 1.53^{a}	36.33 ± 2.08^{a}	NI
Keto.100					40.00 ± 1.00^{a}

(^{a,b,c}) = Means in the same column with different superscripts letter indicates statistically significant differences ($P < 0.05$). Values are mean± standard deviation of triplicate. NI = No inhibition.Diameters of zones inhibition ≥ 10mm exhibited by plant extract were considered active(Usman *et al*.,2008).Standard drugs: Cipr = Ciprofloxacin(Antibacterial drug) , keto=Ketokonazole (Antifugal drug)

Table 4.4 shows zone of inhibitions of methanol extract of young roots. All the organisms were sensitive at all concentration except *Salmonella typhimurium* and *Candida albicansIn* all concentration, *Stahylococcus aureus* showed higher zone of inhibitions while *Escherichia coli* showed lower zones. maximum zone of inhibition (37.33 ± 0.58 mm) was observed in *Staphylococcus aureus* at 100 mg/ml while the minimum zone (10.67 ± 0.58 mm) was observed in *Escherichia coli* at 12.5 mg/ml. the zone of inhibition of extract on *Staphylococcus aureus* was higher compared to the zone of inhibition of ciprofloxacin (35.00 ± 1.00 mm) at the same concentration of 100 mg/ml.

Table 4. 4 Antimicrobial screening of different concentrations of methanol extracts of young root of *Guiera senegalensis.*

Zones of inhibition of micro organisms (mm)

Concentration of extract/Standard drugs (mg/ml)	*Bacillus subtilis*	*Staphylococcus aureus*	*Escharichia Coli*	*Salmonella typhimurium*	*Candida albican*
100	16.00 ± 1.00[b]	37.33 ±5.03[b]	13.33 ± 1.15[b]	NI	NI
50	15.33 ± 0.58[b]	29.67± 2.08[bc]	12.33 ± 0.58[bc]	NI	NI
25	12.00 ± 1.00[b]	26.67 ±5.13[bc]	11.33 ± 0.58[bc]	NI	NI
12.5	11.33 ±0.58[b]	25.33 ±3.06[bc]	10.67 ± 0.58[bc]	NI	NI
Cipr 100	45.33 ±1.53[a]	35.00 ±1.00[a]	22.33 ± 1.53[a]	36.33 ± 2.08[a]	NI
Keto.100					40.00 ±1.00[a]

([a,b,c]) = Means in the same column with different superscripts letter indicates statistically significant differences (P< 0.05). Values are mean± standard deviation of triplicate. NI = No inhibition.Diameters of zones inhibition ≥ 10mm exhibited by plant extract were considered active(Usman *et al*.,2008).Standard drugs: Cipr = Ciprofloxacin(Antibacterial drug) , keto=Ketokonazole (Antifugal drug)

Table 4.5 shows zone of inhibitions of aqueous extract of matured leaves. Zone of inhibition was observed in *Bacillus subtilis* and *Staphylococcus aureus* at all concentration while no zone in other isolates. *Salmonella typhimurium* showed higher zone of inhibitions in all the concentrations, with highest zone of 24.33 ± 3.97 mm at 100 mg/ml while the least zone(11.00 ±00 mm) was *Bacillus subtilis* at 12.5 mg/ml. there was no significant differences in the zones inhibitions of *Bacillus* and *Staphylococcus aureus* at concentrations of 100 mg/ml and 50 mg/ml maximum zone of inhibition showed by *Staphtylococcus aureus* indicates high susceptibility of the isolate.

Table 4.5 Antimicrobial screening of different concentrations of aqueous extracts of matured leaf *Guiera senegalensis*

Zones of inhibition of micro organisms (mm)

Concentration of extract/Standard drugs (mg/ml)	*Bacillus subtilis*	*Staphylococcus Aureus*	*Escharichia Coli*	*Salmonella typhimurium*	*Candida albican*
100	17.33 ± 0.58 [b]	24.33 ± 3.79 [b]	NI	12.00 ± 0.58	NI
50	16.33 ± 0.58 [b]	21.33 ±3.06 [b]	NI	11.00 ± 0.00	NI
25	15.33 ± 0.58 [b]	16.00 ± 1.00[bc]	NI	11.00 ± 1.00	NI
12.5	11.00 ± 00[b]	15.33 ± 0.58[bc]	NI	NI	NI
Cipr 100	45.33 ± 1.53[a]	35.00 ± 1.00 [a]	22.33 ±1.53 [a]	36.33 ± 2.08 [a]	NI
Keto.100					40.00 ±1.00 [a]

([a,b,c]) = Means in the same column with different superscripts letter indicates statistically significant differences (P< 0.05). Values are mean± standard deviation of triplicate. NI = No inhibition.Diameters of zones inhibition ≥ 10mm exhibited by plant extract were considered active(Usman *et al*.,2008).Standard drugs: Cipr = Ciprofloxacin(Antibacterial drug) , keto=Ketokonazole (Antifugal drug)

Table 4.6 shows zones of inhibitions of methanol extract of young leaves. All the extracts concentration shows zone of inhibition against all the isolates except *Staphylococcus aureus* and *Candida albicans*. *Salmonella typhimurim* showed higher zones of inhibitions in all the concentration of the extracts. it showed a maximum zone (26.00 ± 1.00 mm)at concentration of 100 mg/ml while the least zone of inhibition(11.67.00±0.58 mm) was *Bacillus subtilis* at 12.5 mg/ml. The zones of inhibition of *Salmonella typhimurium* at 100mg/ml(26.00±1.00 mm) and 50mg/ml (23.67 ± 67 mm) shows no significant differences. This indicates that *salmonella typhimurium* was more susceptible to the extract.

Table 4.6 Antimicrobial screening of different concentration of aqueous extract of young leaf of *Guiera senegalensis*.

	Zones of inhibition of micro organisms (mm)				
Concentration of extract/Standard drugs (mg/ml)	*Bacillus Subtilis*	*Staphylococcus aureus*	*Escharichia coli*	*Salmonella typhimurium*	*Candida albican*
100	18.00 ± 1.00^{b}	NI	12.00 ± 1.00^{b}	$26\ 0 \pm .1.00^{b}$	NI
50	16.00 ± 1.00^{c}	NI	NI	23.67 ± 0.58^{b}	NI
25	11.67 ± 1.15^{c}	NI	NI	20.33 ± 0.58^{C}	NI
12.5	11.67 ± 0.58^{c}	NI	NI	18.00 ± 1.00^{c}	NI
Cipr 100	45.33 ± 1.53	35.00 ± 1.00	22.33 ± 1.53	36.33 ± 2.08	NI
Keto.100					40.00 ± 1.00

(^{a,b,c}) = Means in the same column with different superscripts letter indicates statistically significant differences ($P < 0.05$). Values are mean± standard deviation of triplicate. NI = No inhibition.Diameters of zones inhibition ≥ 10mm exhibited by plant extract were considered active(Usman *et al*.,2008).Standard drugs: Cipr = Ciprofloxacin(Antibacterial drug) , keto=Ketokonazole (Antifugal drug)

Table 4.7 shows zone of inhibitions of aqueous extract of matured roots. Only *Bacillus subtilis* and *Staphylococcus aureus* showed zone of inhibitions. At concentration of 100 mg/ml *Staphylococcus aureus* showed higher zone of inhibition (18.33 ± 0.58 mm) compared to *Bacillus subtilis* (17.00 ± 1.00) while the least zone(11.33±0.58 mm) was *Bacillus subtilis* at 12.5 mg/ml. *Stahylococcus aureus* showed higher zone of inhibition in all the concentrations of the extract.

Table 4. 7 Antimicrobial screening of different concentrations of aqueous extracts of matured root of *Guiera senegalensis*.

Zones of inhibition of micro organisms (mm)

Concentration of extract/Standard drugs (mg/ml)	*Bacillus subtilis*	*Staphylococcus Aureus*	*Escharichia Coli*	*Salmonella typhimurium*	*Candida albican*
100	17.00 ± 1.00[b]	18.33 ± 0.58[b]	NI	NI	NI
50	16.00 ± 1.00[b]	16.33 ±1.00[b]	NI	NI	NI
25	13.00 ± 1.73[c]	15.67 ± 0.58[c]	NI	NI	NI
12.5	11.33 ± 0.58[c]	12.00 ± 1.00[c]	NI	NI	NI
Cipr 100	45.33 ± 1.53[a]	35.00 ± 1.00[a]	22.33 ± 1.53[a]	36.33 ± 2.08[a]	NI
Keto.100					40.00 ± 1.00[a]

([a,b,c]) = Means in the same column with different superscripts letter indicates statistically significant differences (P< 0.05). Values are mean± standard deviation of triplicate. NI = No inhibition.Diameters of zones inhibition ≥ 10mm exhibited by plant extract were considered active(Usman *et al*.,2008).Standard drugs: Cipr = Ciprofloxacin(Antibacterial drug) , keto=Ketokonazole (Antifugal drug)

Table 4.8 shows zone of inhibitions of aqueous extract of young roots. *Bacillus subtilis* and *staphylococcus aureus* showed zone of inhibitions at all concentration while *Escherichia coli, Salmonella typhimurium* and *Candida albicans* were not susceptible to the extract. At 100 mg/ml concentration, zone of inhibition of *Bacillus subtilis* (18.67 ± 1.53 mm) and *Staphylococcus aureus* (17.33 ±1.53 mm) were not statistical different. Both isolates showed least zones of inhibitions of 11.33 ± 0.58 mm and 11.67 ± 0.58 mm at concentration of 12.5 mg/m for *Bacillus* and *Staphylococcus aureus respectively*

Table 4.8 Antimicrobial screening of different concentrations of aqueous extracts of young root of *Guiera senegalensis.*

Zones of inhibition of micro organisms (mm).

Concentration of extract/ Standard drugs (mg/ml)	*Bacillus subtilis*	*Staphylococcus Aureus*	*Escharichia Coli*	*Salmonella typhimurium*	*Candida albican*
100	18.67 ± 1.53^{b}	17.33 ± 1.53^{b}	NI	NI	NI
50	15.67 ± 0.58^{c}	16.00 ± 1.00^{b}	NI	NI	NI
25	12.00 ± 1.00^{C}	12.67 ± 1.53^{bc}	NI	NI	NI
12.5	11.33 ± 0.58^{b}	11.67 ± 0.58^{bc}	NI	NI	NI
Cipr 100	45.33 ± 1.53^{a}	35.00 ± 1.00^{a}	22.33 ± 1.53^{a}	36.33 ± 2.08^{a}	NI
Keto.100					40.00 ± 1.00^{a}

(^{a,b,c}) = Means in the same column with different superscripts letter indicates statistically significant differences ($P < 0.05$). Values are mean± standard deviation of triplicate. NI = No inhibition.Diameters of zones inhibition ≥ 10mm exhibited by plant extract were considered active(Usman *et al*.,2008).Standard drugs: Cipr = Ciprofloxacin(Antibacterial drug) , keto=KetoConazole (Antifugal drug)

4. 2 Minimum inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The minimum inhibitory concentration (MIC) was determined as the lowest concentration of test samples that resulted in a complete inhibition of visible growth in the broth. Following anaerobic incubation of MIC plates, the minimum bactericidal concentration (MBC) was determined on the basis of the lowest concentration of the methanol and water extracts that kill 99.9 % of the test bacteria.

Table 4.9 and 4.10 shows Minimum inhibitory concentration MIC and Minimum Bactericidal concentration MBC of methanol and aqueous extracts.

The minimum inhibitory concentration (MIC) was determined as the lowest concentration of test samples that resulted in a complete inhibition of visible growth in the broth. Following anaerobic incubation of MIC plates, the minimum bactericidal concentration (MBC) was determined on the basis of the lowest concentration of the methanol and water extracts that kill 99.9 % of the test bacteria. Both extracts exhibited low minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against all the test isolates. Most exhibited MIC at concentration range of 6.25 mg/ml to 12.5 mg/ml except *Escherichia coli* and *Salmonella tphimurium* at 25mg/ml as showed table 4.10. methanol extract of young leaf showed the lowest MIC, 3.125 mg/ml against *Staphylococcus aureus* .

Most extracts exhibited MBC at a low concentration of 12.5 mg/ml to 25 mg/ml against all isolates except *E. coli* whose MBC was exhibited at 50 mg/ml. The lowest MBC was observed in methanol extract of young and matured leaves at 6.25 mg/ml and 3.125 mg/ml against *Staphylococcus aureus* respectively

Table 4 .9 Mininmum inhibitory concentration (MIC) of methanol and aqueous extracts of leaf and roots of *Guiera senegalensis* (mg/ml)

Bacteria isolates	MT matured leaves(mg/ml)	MT young leaves(mg/ml)	MT matured roots(mg/ml)	MT young roots(mg/ml)	AQ matured matured(mg/ml)	AQ young leaves(mg/ml)	AQ matured roots(mg/ml)	AQ young roots(mg/ml)
Bacilus subtilis	12.5	12.5	12.5	25	6.25	12.5	6.25	12.5
Staphylococcus aureus	12.5	3.125	12.5	6.25	6.25	_	6.25	12.5
Escherichia coli	25	50	_	12.5	_	50	_	_
Salmonella typhimurium	25	_	_	25	_	_	_	_

Key: AQ = Aqueous extract, MT = Methanol extract, - = No minimum inhibitory concentration (MIC)

Table 4.10 Mininmum bactericidal concentration (MBC) of methanolic and aqueous extracts of leaf and root of *Guiera senegalensis* (mg/ml)

Bacteria isolates	AQ matured leaves	AQ young leaves	AQ matured roots	AQ young roots	MT matured leaves	MT young leaves	MT matured roots	MT young roots
Bacilus subtilis	12.5	25	12.5	25	25	25	25	50
Staphylococcus aureus	12.5	_	12.5	25	25	3.125	25	12.5
Escherichia coli	_	100	_	_	50	100	_	12.5
Salmonella typhimurium	_	50	_	_	50	_	_	50

Key: AQ = Aqueous extract, MT = Methanol extract, - = No Minimum bactericidal concentration(MBC)

4.3 Antimicrobial screening of crude and protein fractions of the leaf and root of *G. senegalensis*.

The crude protein of the leaves showed activity against Gram positive bacteria only while the roots showed lower zones of inhibitions against the isolates. Higher zone of inhibitions (21.00±1.00 and 18.33±1.58) were observed at concentration of 11.22 mg/ml and 14.50 mg/ml for matured and young leaves respectively. The fractions were active against Gram positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*).*Staphylococcus aureus* showed higher zone of inhibitions in the young leaves fractions concentration of 1.20mg/ml(19.67±0.58mm),followed by matured leaves 1.10mg/ml(16.67±0.58 mm) while the least zones of (13.00±1.00 mm) and (15.00±0.58 mm) were observed in *Bacillus subtilis* for matured and young leaves fractions respectively.

Table 4.11 Purification of Bioactive protein of the leaf of young and matured *Guiera senegalensis.*

Purification steps	Total protein(mg/ml)	Total activity(AU)	Specific Activity(AU/mg)	Recovery (%)	Purification fold
Matured leaves					
Crude protein	14.5	3500	241.38	100	1
80% ammonium sulfate precipitation	8.35	2700	323.35	77.14	1.34
Gel filtration Sephadax	1.2	1500	1250	51.43	5.18
young leaves					
Crude protein	15.1	4580	303.31	100	1
80% ammonium sulfate precipitation	8.25	4300	521.21	93.89	1.72
Gel filtration Sephadax	1.1	3100	2818.18	67.69	9.29
Matured roots					
Crude protein	9.04	2500	276.55	100	1
80% ammonium sulfate precipitation	6.5	2300	352.85	92	1.28
Gel filtration Sephadax	2	2100	1050	84	3.80
Young roots					
Crude protein	11.22	2800	249.55	100	1
80% ammonium sulfate precipitation	6.74	2600	385.76	92.86	1.55
Gel filtration Sephadax	1.1	2000	1818.18	71.43	7.29

Table 4.12 Antimicrobial activity of the leaf and root of the crude and partial purified protein fractions of *Guiera senegalensis*

Samples	*Bacillus subtilis*	*Staphylococcus aureus*	*Escherichia Ecoli*	*Salmonella typhimurium*	*Candida albicans*
Crude extract of matured leaves	++	+++	_	_	_
Crude extract of young leaves	++	+++	_	_	_
Crude extract of matured roots	+	+	_	_	_
Crude extract of young root	+	+	_	_	_
Peak of17th fraction of gel filtration of matured leaves	++	++	_	_	_
Peak of 27th Fraction of gel filtration of young leaves	++	++	_	_	_
Peak of 20th fraction of gel filtration of matured root	_	+	_	_	_
Peak of 24th fraction of gel filtration of young root	_	+	_	_	_

Key: +++ = very strong activity, ++ = strong activity, + = activity present, –– = activity

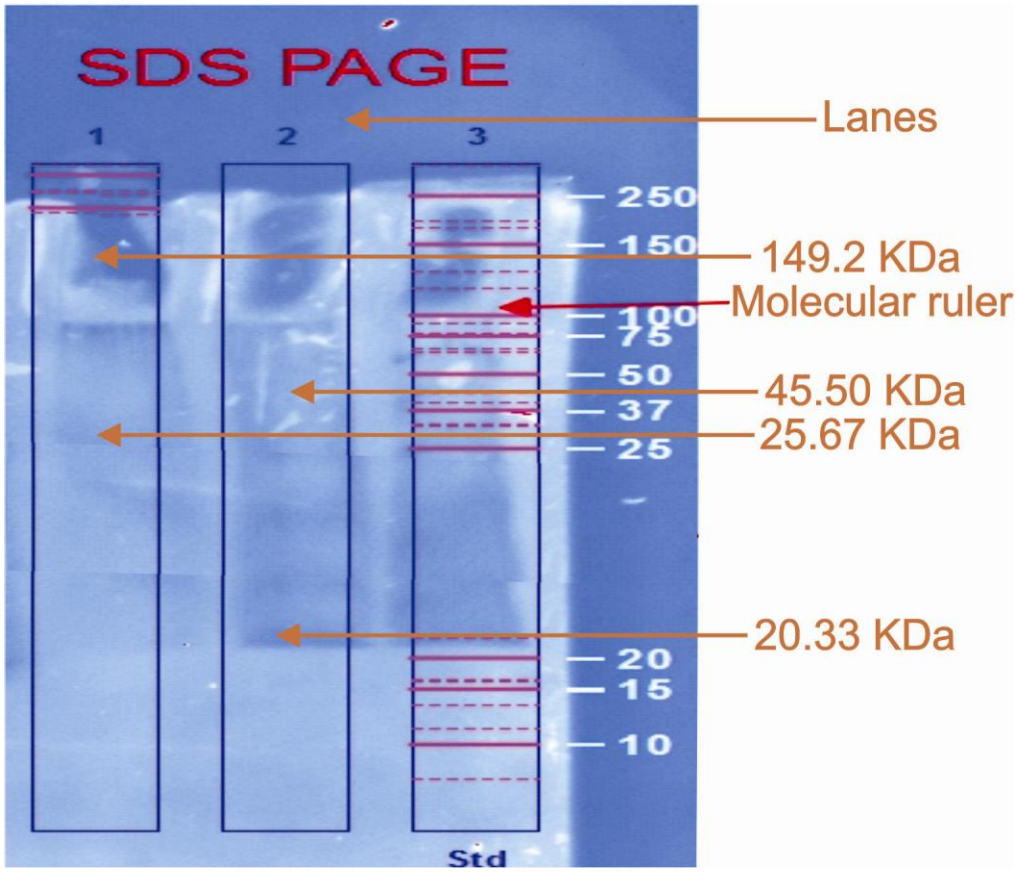


Figure 4. 4 SDS PAGE of Young and Matured Leaf of *Guiera senegalensis*

Lane 1 (partial purified fraction of matured leaf)

Lane 2 (partial purified fraction of young leaf)

Lane 3 protein standard.

4.4 The Amino Acid Profile of Leaf and Root of Young and Matured *Guiera senegalensis*.

Seventeen (17) amino acids were obtained namely; lyisine, histidine, arginine, cysteine , valine, methionine, isoleucie, glutamic acid, proline, glycine, alanine, leucine, tyrosine, serine, threonine, aspartic acid and phenylalanine. The analysis showed that, young leaf fraction has higher values of amino acids compared to the fractions of matured leaf and roots. Glutamic acid showed maximum concentration followed by aspartic acids and leucine in both leaves and roots of *G. senegalensis.*

In matured leaf fraction, high amount of glutamics acid (9.39 g/100g of protein), followed by aspartic acids (9.29 g/100g of protein), .leucine (5.71 g/100g of protein), arginine (4.25 g/100g of protein) while cysteine, methionine and tyrosine have the least concentration of 0.93g,1.09g and 1.93 g/100g of protein respectively. In young leaf fraction, glutamic acid has high amount of 10.58 g/100g followed by aspartic acids (9.47 g), leucine (6.48 g), arginine(4.93 g) and the least were cysteine, methionine, and tyrosine have low amount of 1.19 g, 1.30 g and 2.32 g/100g of protein respectively. In young root and matured root, glutamic acid was 5.66 g and 6.26 g/100g of protein respectively, followed by leucine 3.68 g and 3.29 g/100g of protein respectively while the least concentration of amino acid in young root was cysteine (0.75 g/100g of protein) and matured root was proline (0.53 g/100g of protein). Some amino acids occurred in lower or higher relative amounts of the samples. For instance, there was significant less glycine, cysteine methionine and more of glutamic acid, aspartic acids in both leaf and root

Table 4.13 Amino Acids Profile of the Actively Partially Purified Protein of the leaf and Root of Young and Matured *Guiera senegalensis.*

Amino acids	Matured leaves (g/100g 0f protein)	Young leaves (g/100g 0f protein)	Matured roots (g/100g 0f protein)	Young roots (g/100g 0f protein)
Lysine	3.7	4.62	3.38	2.63
Histidine	2.26	2.32	0.69	0.81
Arginine	4.25	4.93	2.21	1.62
Aspartic acid	**9.29**	**9.47**	**2.62**	**3.43**
Threonine	2.61	3.22	1.11	0.89
Serine	2.97	3.72	1.02	2.32
Glutamic acid	**9.39**	**10.55**	**6.26**	**5.66**
Proline	3.18	4.14	0.53	1.27
Glycine	3.31	3.6	1.21	2.33
Alanine	4.09	4.48	0.93	0.77
Cystein	0.93	1.19	0.79	0.753
Valine	3.83	4.07	2.61	3.19
Methionine	1.09	1.3	0.62	0.52
Isoleucine	2.82	3.58	1.69	1.25
Leucine	5.71	6.48	3.29	3.68
Tyrosine	1.93	2.41	1.61	1.29
Phenylalanine	4.06	4.48	2.7	2.2

CHAPTER FIVE

DISCUSSION

In this study, the amino acid profile of the leaf and root of young and matured *Guiera senegalensis* were analysed. Seventeen (17) amino acids were obtained namely lyisine, histidine, arginine, cysteine , valine, methionine, isoleucine, glutamic acid, proline, glycine, alanine, leucine,tyrosine, serine, threonine, aspartic acid and phenylalanine. The values are within the ranges of reported values for some antimicrobial amino acid reported by Dowling *et al.*,(2011) that 17 % of the amino acids in plant antimicrobial peptides are positively or negatively charged. Specifically, arginines and/or lysines comprise more than 70 % of all charged residues found in these peptides, while the remaining 30 % consists of the negatively charged aspartic acid and glutamic acid (Dowling *et al.,* 2011). Young leaf showed higher values of amino acids compared to the matured leaf and roots. Some amino acids occurred in lower or higher relative amounts of the samples. For instance, there was significant less glycine, cysteine, methionine and more of glutamic acid, aspartic acids in both leaves and roots. The appreciable amount of acidic amino acids may be responsible for the activity of the protein fraction against the Gram positive bacteria tested.

The antimicrobial activity of the following test isolates "*Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Salmonella typhimurium* and *Candida albicans"*indicates that both methanol and aqueous extracts were effective against most of the test isolates compared to crude protein which was effective only to Gram positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*).All extracts were ineffective against the fungus; *Candida albicans.* According to

chemical and laboratory standard institute CLSI (2005), any plant material should be considered an effective therapeutic agent if its extract produces zones of inhibition ≤ 15.00 mm on the target pathogenic organism. Activity of plant extracts to test bacteria is normally expressed invitro as zone of inhibition in millimeter (≤10.00 mm) is regarded as effective zones. (Usman, 2008).

Generally, both extracts showed a wide range of antimicrobial activity when compared to the positive control but there was a slight difference between the extracts with respect to the plant's part. Methanol extract of matured leaf of *G. senegalensis* showed activity against all the bacteria isolates while aqueous extract of same leaf was active against three isolates; *Bacillus subtilis Staphylococcus aureus and salmonella typhimurium* .Methanol extract of young leaf showed higher activity against three (3) isolates except *Salmonella typhimurium* while the aqueous extract of same leaf was effective against three isolates except *Staphylococcu aureus*. Methanol and aqueous extracts of matured roots showed antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus.* Similarly methanol extract of young root was also active against *Bacillus subtilis* and *Staphylococcus aureus* while the aqueous extracts of same root was effective against *Bacillus subtilis, Staphylococcus aureus* and *Escherichia coli.* Both methanol and aqueous leaves extracts showed wide range of antibacterial activity which agreed with similar reports documented by Umeh *et al., (*2005).That the antimicrobial constituents of plants are preferentially concentrated in the leaves. In this work, the leaf extract showed a higher percentage of growth inhibition than the root of the plant. The observed antimicrobial effects on the isolates is believed to be due to the presence of tannins, flavonoids and saponins which have shown to posses antimicrobial properties(Yagana *et al* ., 2012).Some workers have also

attributed the observed antimicrobial effect of plants extracts to the presence of these secondary metabolites(Radulovic *et al.,* 2013). As shown by the phytochemical screening results, the various extracts of G. senegalensis contain all these secondary plant metabolites. Some other workers have identified, that tannins, flavonoids and alkaloids in the extracts of of some medicinal plants example Euphorbia hirta posses antimicrobial activity(Yadav *et al.,* 2011).thus,the growth inhibition effect of the extracts on the micro organism could be attributed to the presence of bioactive substances such as phenolic acids, tannins and flavonoids as reported by other workers. Phenolic acids are highly hydroxylated phenols, scientific evidence show that increase hydroxylation of phenol result to increased toxicity to pathogens (Yadav *et al.,* 2011)

The zone of inhibition for methanolic extracts of young leaf and root (42.00 ± 1.00 mm and 37.33 ± 5.03 mm respectively) at a concentration of 100 mg/ml was more than that of the standard drug ciprofloxacin(35.00 ± 1.00 mm) against *Staphylococcus aureus* of same concentration. This result is in accordance with the experiment carried out by (Aiylaagbe *et al*., 2007).

The crude protein of the leaf and root showed activity against Gram positive bacteria only. The fractions were active against Gram positive bacteria (*Bacillus subtilis and Staphylococcus aureus*). *Staphylococcus aureus* showed higher zone of inhibitions (19.67 ± 0.58 mm) in the young leaf fractions at a concentration of 1.20 mg/ml.

The molecular weight of the partial purified proteins of the matured leave were 25.67 kDa and 149.2 kDa at protein concentrations of 1.10 mg/ml while the young leaves were 20.33k Da and 45.5 kDa at protein concentration of 1.20 mg/ml.These antimicrobials proteins falls within the range of reported antimicrobial proteins such as Glycine-Rich protein(30 kDa),contains 70 % glycine residues, plant lectin (140 kDa), Kunitz proteinase inhibitors(26k Da) which is rich in cystein and 2S Albumin(26 kDa) rich in glutamic acids.the antimicrobial efficiency depends on several characteristics of the protein or peptide including molecular mass, sequence, charges, conformation, secondary and tertiary structures, presence or absence of disulfide bonds and hydrophobicity(Cândido *et al.*, 2011). The resistance of Gram negative could be due to the fact that they have an additional outer phospholipids membrane that makes the cell wall impermeable to lipophilic and hydrophilic solutes (Manikandan *et al.*, 2011).

The minimum inhibitory concentration (MIC) was determined as the lowest concentration of test samples that resulted in a complete inhibition of visible growth in the broth. Following anaerobic incubation of MIC plates, the minimum bactericidal concentration (MBC) was determined on the basis of the lowest concentration of the methanol and water extracts that kill 99.9 % of the test bacteria. Both methanol and water extracts exhibited low minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against all the test isolates. Most exhibited MIC at concentration range 6.25 mg/ml to 12.5 mg/ml except *Escherichia coli* and *Salmonella typhimurium* at 25 mg/ml. Methanolic extract of young leaf showed the lowest MIC, 1.563mg/ml against *Staphylococcus aureus.* Pure protein fraction of the young leaves showed more activity on *Staphylococcus aureus* compared with other fractions.

Most extracts exhibited MBC at a low concentration of 12.5 mg/ml to 25 mg/ml against all isolates except *E.coli* whose MBC was exhibited at 50 mg/ml. The lowest MBC was observed in methanolic extract of young and matured leaves at 6.25 mg/ml and 3.125 mg/ml against *Staphylococcus aureus* respectively. The low MIC and MBC exhibited by the extracts against *Staphylococcus aureus, E. coli, Salmonella typhimurium* and *Bacillus subtilis* are of great significance in the health care delivery system, since it could be used as an alternative to orthodox antibiotic in the treatment of infections caused by these microbial pathogens, especially as they frequently developed resistance to known antibiotics.

The presence of these biologically active chemicals and antimicrobial amino acids may have been responsible for the antimicrobial activity of these plant extracts. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls and disrupts microbial membranes (Temitayo *et al.,* 2012).

Saponins are surface active agents which alter the permeability of the cell wall of organisms thus facilitating the entry of toxic materials or leakage of vital constituents from the cell (Daniyan *et al*., 2010). In medicine, saponins are used as hypercholesterolemia, hyperglycemia, antioxidant, anti-cancer, anti- inflammatory agents due to their detergent property (*Ngbede et al.,* 2008). These properties confirm saponins as potent antimicrobial agent.

Tannins are polyphenols known to exhibit antibacterial, antiviral and anti-tumor activities. It was also reported that certain tannins are known to inhibit HIV replication selectively and is also used as diuretic (Evans, 2002). The leaves and roots of *G. senegalensis* also showed the

presence of alkaloid which is known to possess anti-inflammatory and anti-asthmatic actions. This confirms the use of *G. senegalensis* in folklore medicine for the treatment of malaria. Alkaloid is also one of the largest groups of phytochemicals that has amazing effect on humans which led to the development of powerful pain killer medications (Staerk *et al*., 2002). Flavonoids have been referred to as nature's biological response modifiers because of strong experimental evidence of their inherent ability to modify the body's reaction to allergen, virus and carcinogens. Some flavonoids have also been reported to act like some coumarins in the inhibition of giant cell formation in HIV infected cell cultures (Evans, 2002).

The inhibitory activity exhibited by the secondary metabolites tends to agree with the reports of Ashish *et al*., (2013) that linked the antimicrobial of plants to the presence of secondary metabolites. Also, the presence of appreciable amount of antimicrobial acidic amino acids may contribute to the antimicrobial activity of the protein fractions against Gram positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus.* This inhibitory activity agreed with a report of Zhang *et al.,* (2011) on antimicrobial peptide of *Pichia pastoris.*

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATION.

6.1 SUMMARY

The results of the study showed that all the extracts of *Guiera senegalensis were active against: Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Salmonella typhimurium* but inactive against *Candida albican.*

The methanolic extracts was more active than the aqueous extract , the young leaf and young root proved to be more potent than the matured leaf and root which agreed with antimicrobial potential of *Ricinus communis* plant by Naz *et al.*, *(*2012).

Protein partially purified proteins were active against *Bacillus subtilis* and *Staphylococcus aureus* (Gram positive bacteria).

Guiera senegalensis contain appreciable amount of amino acids, more was observed in the partially purified protein of the young leaf

The molecular weight of the partial purified proteins of the matured leave were 25.67 kDa and 149.2 kDa at protein concentrations of 1.10 mg/ml while the young leaves were 20.33 kDa and 45.50 kDa at protein concentration of 1.20 mg/ml.

Methanol extracts of matured leaf and root of *G. senegalensis* showed high amount of saponins and flavonoids compared with the methanol extracts of the young leaf and root. The present of

low oxalates, tannins, phytate and cyanogenic glycosides also support the safety use of the plant since they are within the range of reported values for leafy vegetables(Kubmarawa *et al*.,2008).

6.2 CONCLUSION

The leaf and root of *Guiera senegalensis* possesses antimicrobial activity against pathogenic bacteria and may be used in susceptibility cases. These extracts could be used as alternative for commercial orthodox antibiotics for treatment of antimicrobial infections.

6.3 RECOMMENDATION.

1. The leaf and root of *Guiera senegalensis* could be used in the treatment of some diseases caused by bacterial, most especially disease caused by *Staphylococcus aureaus*. Further studies are required to advocate its systemic use in infectious diseases.

2. The amino acids sequence should be carried out on the young leaf that was more susceptible to the test isolates.

3. Purification of the extract is also recommended in other to obtain the pure bioactive components for pharmaceutical and other industrial uses.

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APPENDIX I.

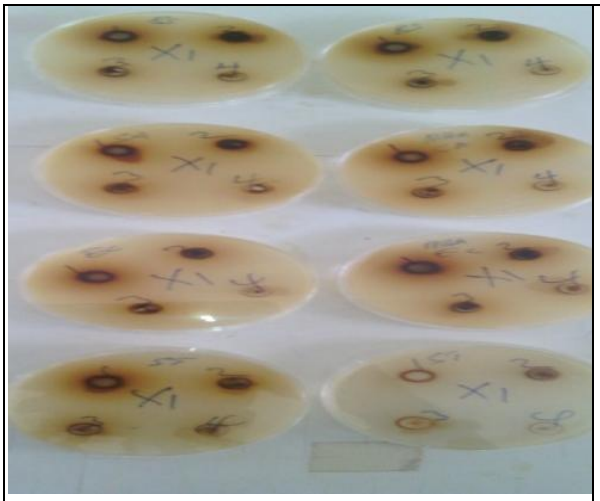
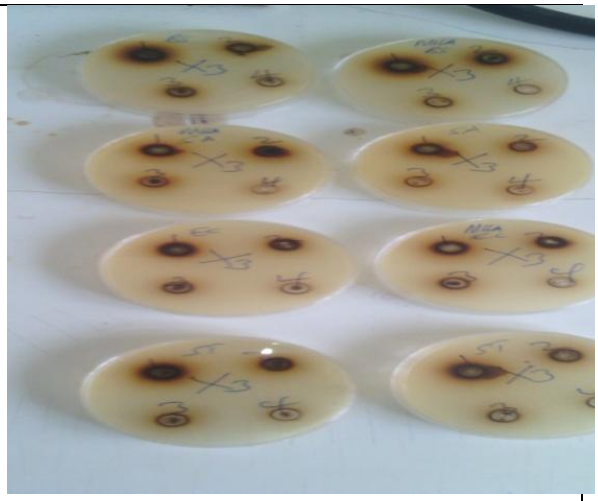
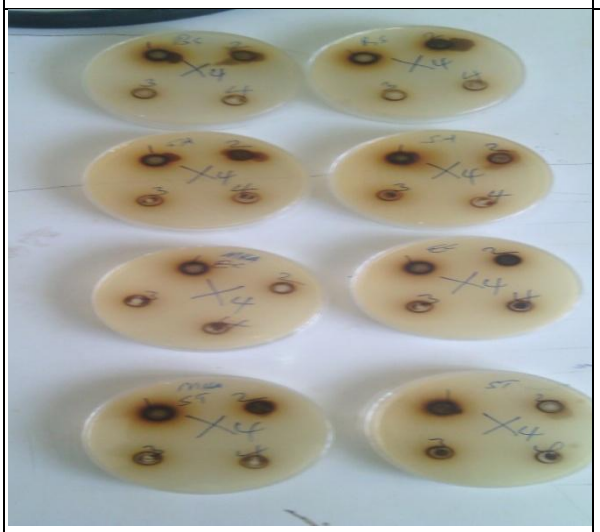
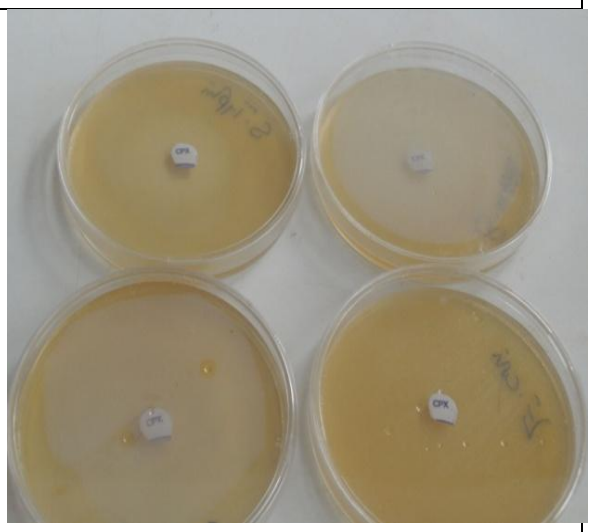
	
Cultured plates for aqueous extract	**cultured plates for metanolic extract**
	
Cultured plates for protein extract	**Standard antibiotic plates**

Plate 1. Antimicrobial cultured plates of the leaves and roots extracts of *Guiera ssenegalensis.*

APPENDIX ii.

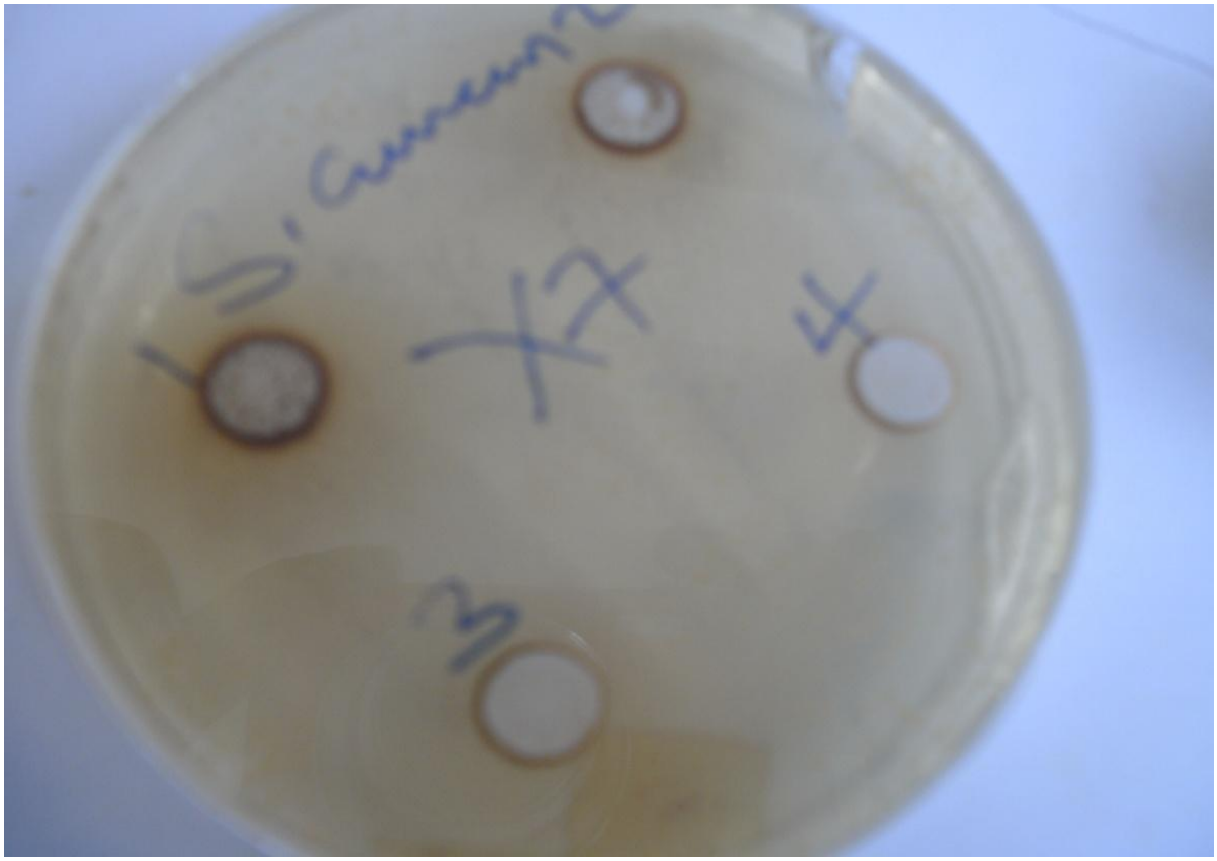


Plate 2: Plate of matured (3) and young(4) leaves of methanolic extracts that showed maximum zones of inhibition against *Staphylococcus aureus.*

Appendix iii



Plate3: Plate of the most active protein fractions of young (3) and matured (4) leaves of *G. senegalensis* that showed maximum zone of inhibition against *Staphylococcus aureus*

Appendix iv

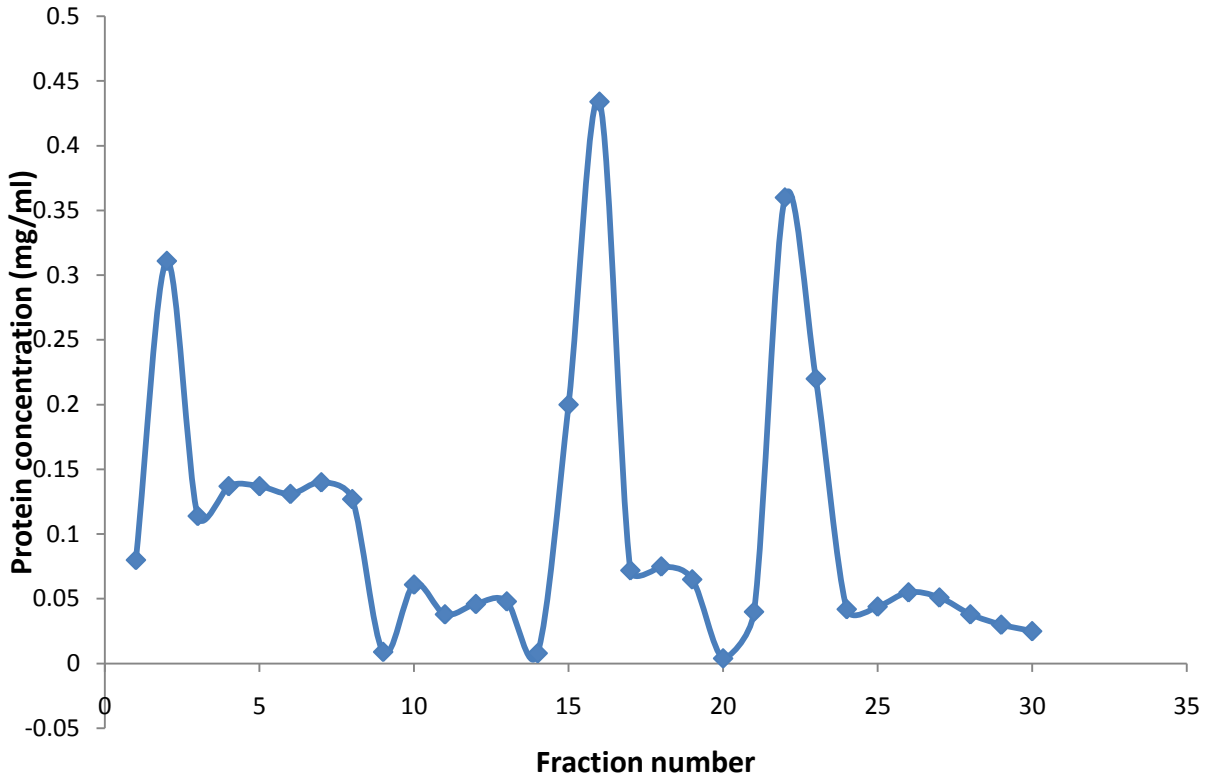


Figure 4.1 Gel filtration pattern of matured leaf of *Guiera senegalensis.*

Appendix v

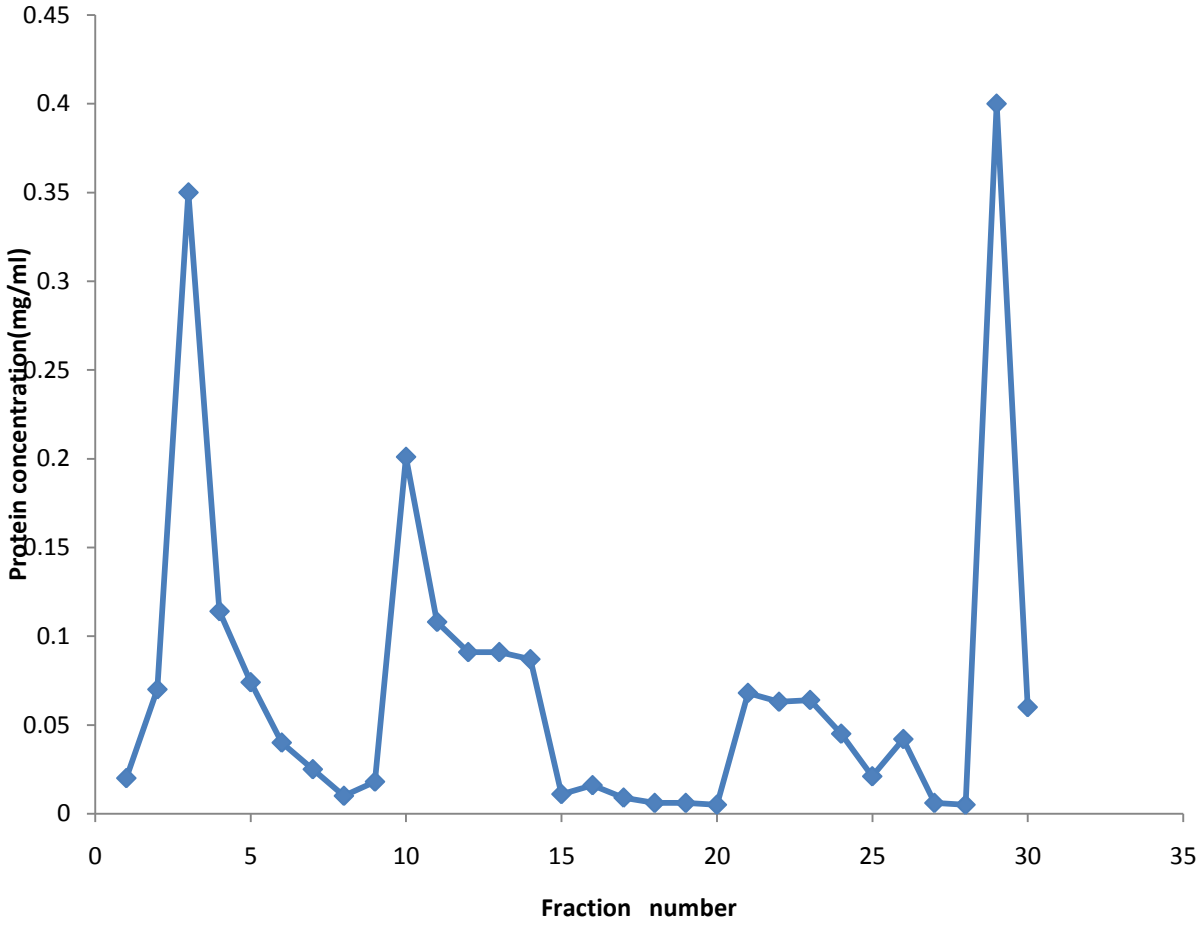


Figure 4.2 Gel filtration pattern of young leaf of *Guiera senegalensis.*

Appendix vi

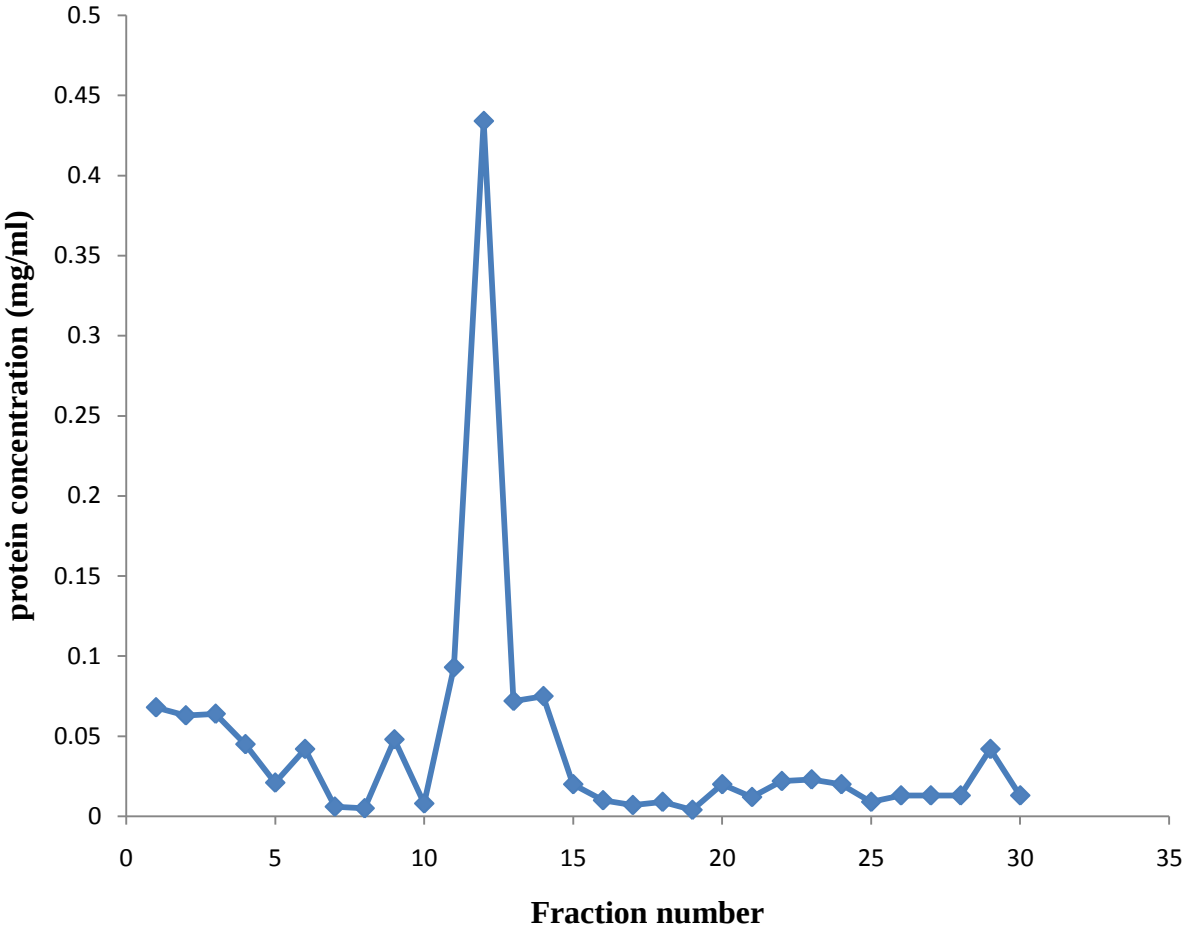


Figure 4.3 Gel filtration pattern of matured root of *Guiera senegalensis*.

Appendix vii

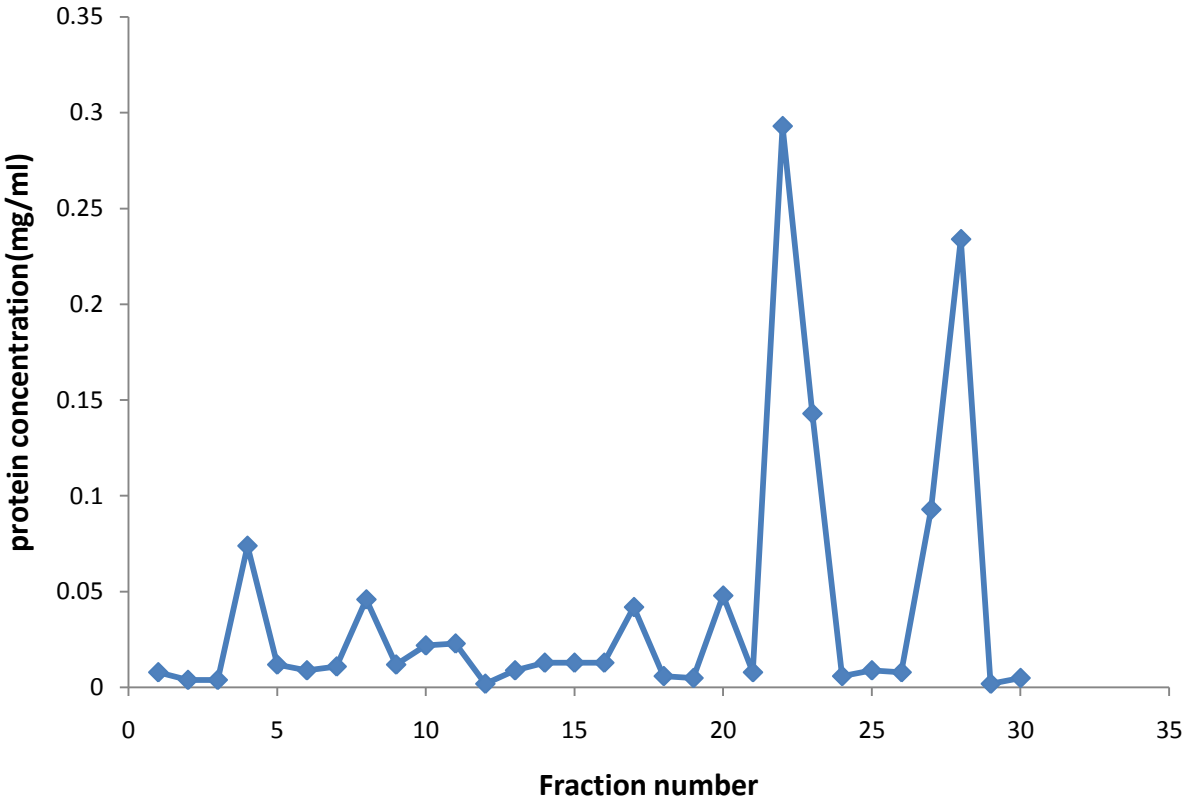
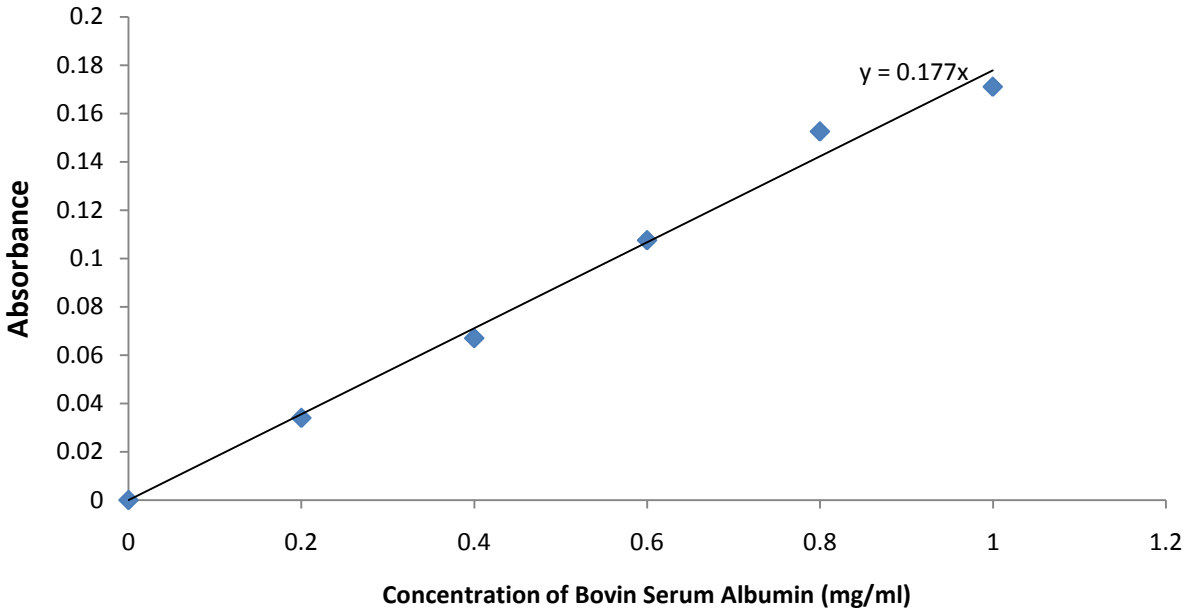


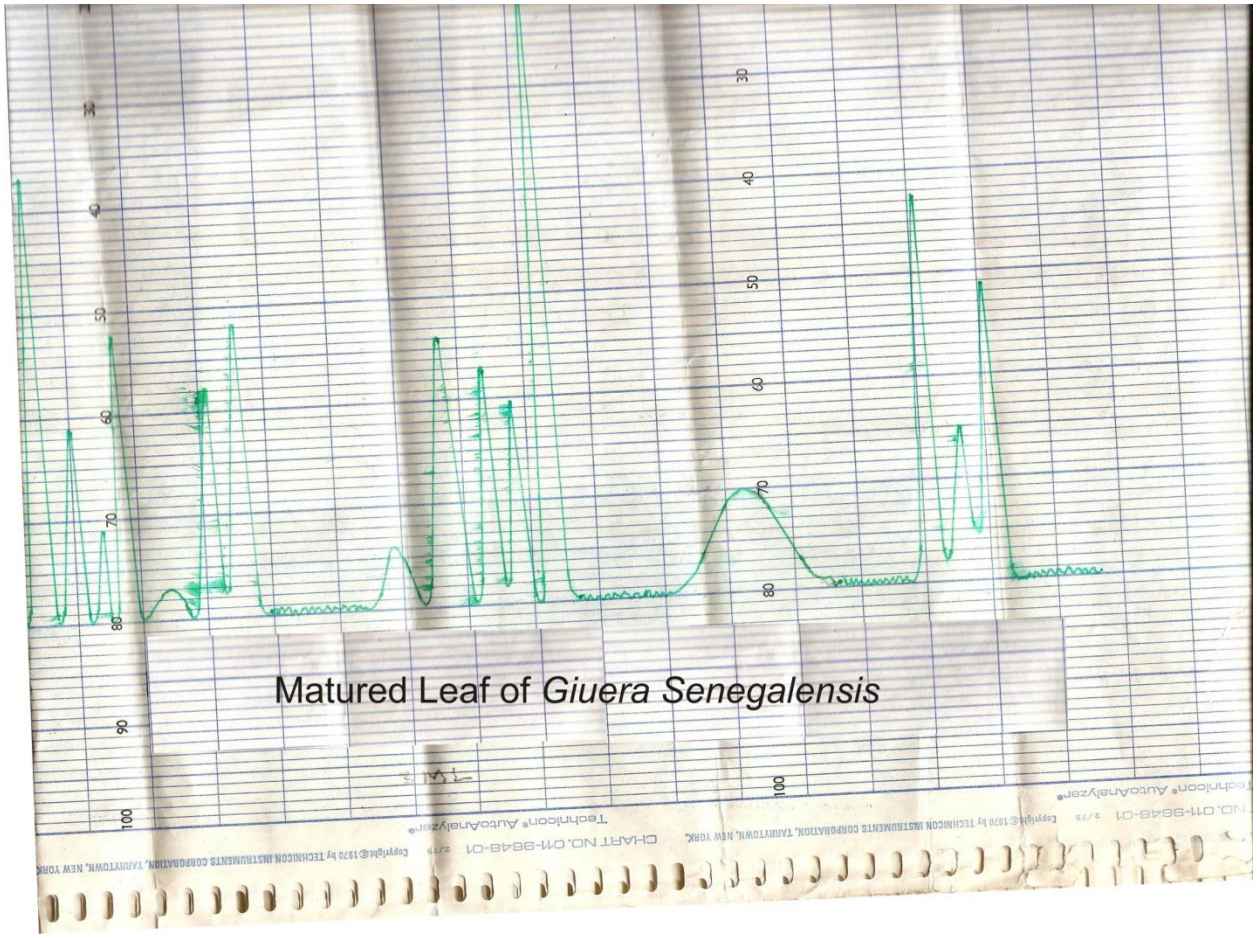
Figure 4.3 Gel filtration pattern of young root of *Guiera senegalensis.*

Appendix viii



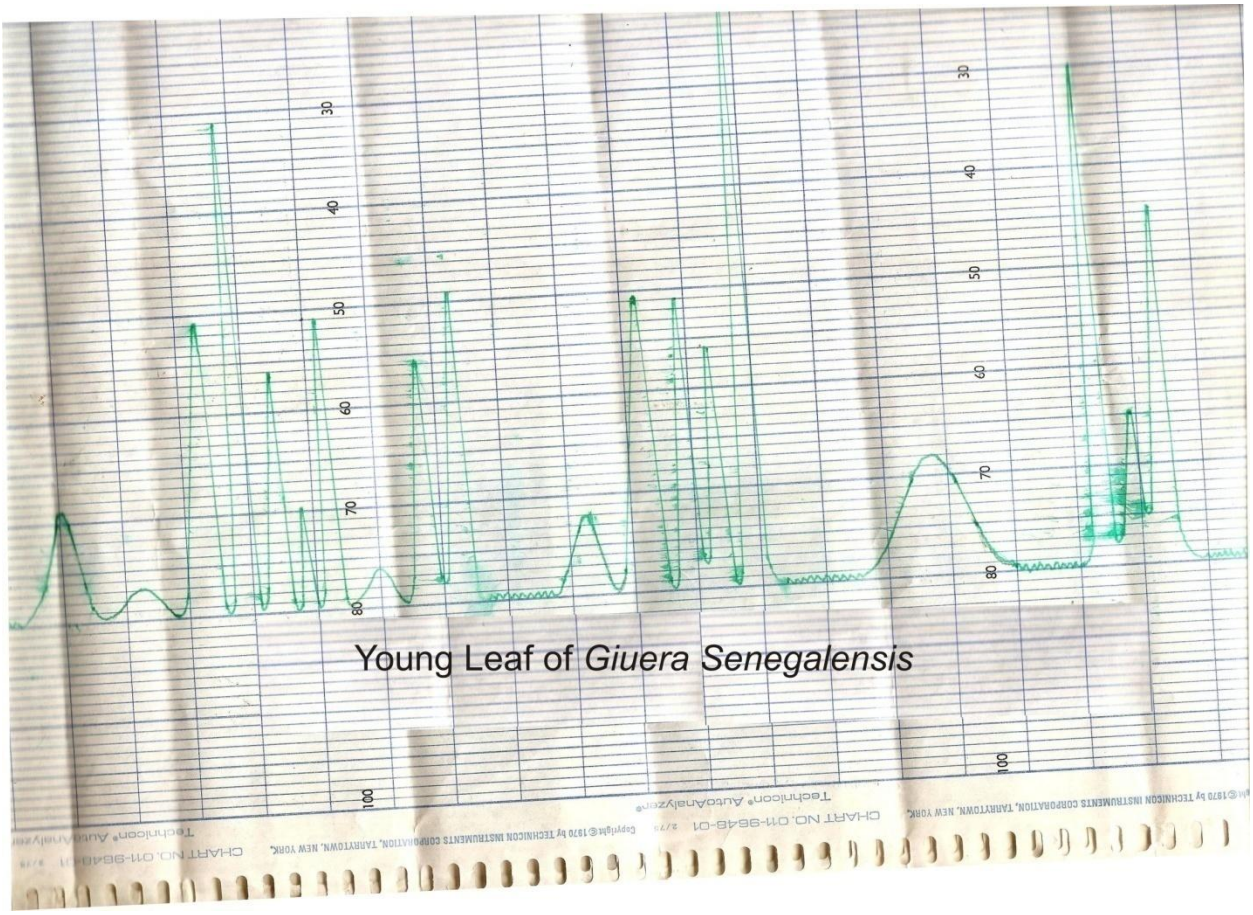
Standard Curve for determination of protein concentration

Appendix ix



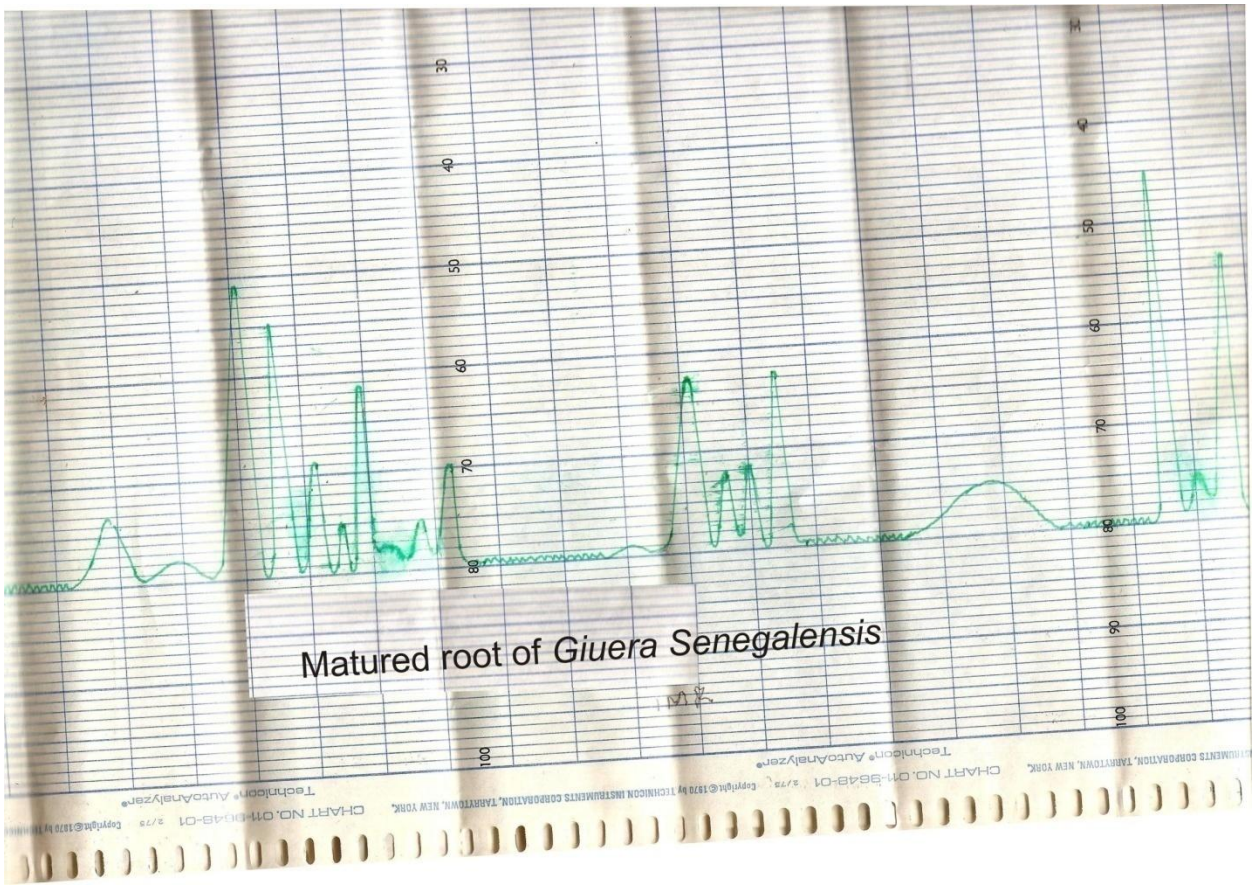
Graph of amino acids profile of matured leaf of *Guiera senegalensis*

Appendix xi



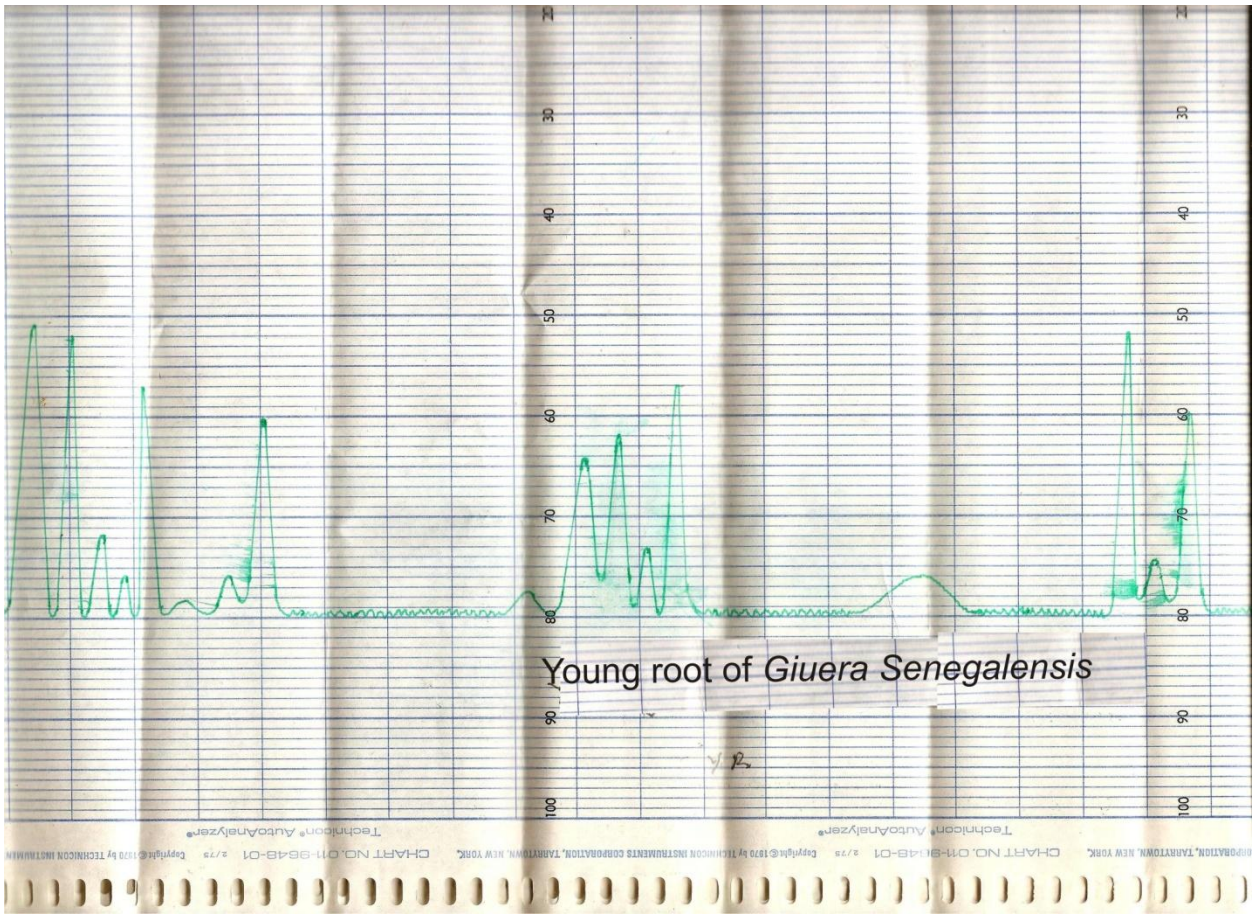
Graph of amino acids profile of young leaf of *Guiera senegalensis*

Appendix xii



Graph of amino acids profile of matured root of *Guiera senegalensis*

Appendix xiii



Graph of amino acids profile of young root of *Guiera senegalensis*