ANTI-MICROBIAL ACTIVITY, TOXICITY AND CHEMICAL CHARACTERIZATION OF EXTRACTS OF *Indigofera lupatana* BAKER F. PLANT

SOSPETER NGOCI NJERU

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EGERTON UNIVERSITY

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DECLARATION AND RECOMMENDATION

Declaration

This thesis is my original work and has not been presented, wholly or in part, for an award of degree in any other university.

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Sospeter Ngoci Njeru.

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Recommendation

We wish to confirm that this thesis was carried out under our supervision and has our approval to be presented for examination as per the Egerton University regulations.

Signature…….Date……

Prof. Matasyoh J.C.

Department of Chemistry

Egerton University

Signature……...…Date……

Dr. Mwendia C. M.

Department of Biochemistry and Molecular Biology

Egerton University

Signature……...…Date……

Dr. Mwaniki C. G.

Department of Biochemistry and Molecular Biology

Egerton University

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DEDICATION

This work is dedicated to my parents; Humphrey Njeru and Margaret Njeru, my wife Abishag Ngoci, my sister Flora Muthoni; Luke, Deborah and other siblings. Your constant inspiration, encouragements and support made this work successful.

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ABSTRACT

Indigofera lupatana Baker F. (locally known as *Mugiti*) has been used by Mbeere community of Kenya to treat cough, diarrhea, pleurisy and gonorrhea. These and other infectious diseases are a cause of morbidity and mortality in humans and animals. Their effects are further aggravated by drug resistance. There is also increased emergence and re-emergence of infections from previously harmless micro-organisms, as part of nosocomial and opportunistic infections. This calls for search of new drugs that will mitigate these problems. Indigenous plants are promising as a cheap alternative source of new therapeutic agents. Although the use of phytomedicine has been in practice for long, little has been done to evaluate their effectiveness, safety, target organisms and also their chemical characterization. Therefore, the aim of this study was to investigate the antimicrobial activity, toxicity and phytochemical screening of root extracts of *Indigofera lupatana* Baker F. Powdered sample of *I. lupatana* Baker F. roots were sequentially extracted using hexane, ethyl acetate, dichloromethane and methanol solvents. The resultant fractions were subjected to anti-bacterial assay, phytochemical tests and brine shrimp toxicity bioassay. The fractions showed the highest activity against *Bacillus subtilis* (28.5 ± 0.3mm), *Staphylococcus aureus* (22.6 ± 1.0mm), *Bacillus cereus* (22.0 ± 0.3mm), *Escherichia coli* (21.7 ± 0.7mm), *Pseudomonas aeruginosa* (21.5 ± 0.9mm), *Salmonella typhimurium* (17.3 ± 0.3mm), *Klebsiella pneumonia* (15.3 ± 0.4mm) and *Proteus mirabilis* (12.3 ± 0.5mm). The activity was greater among the Gram positive bacteria than Gram negative bacteria. Activity of the ethyl acetate, dichloromethane and methanol extracts on *bacillus cereus* species and that of ethyl acetate on *P. aeruginosa* was similar to the activity of chloramphenicol drug (with $P > 0.05$). The MIC ranged from between 21.9 to over 750mg/ml. Phytochemical testing revealed presence of Phenolics, Flavonoids, Tannins, Saponins, Terpenoids, Cardiac glycosides, Steroids and Phlobatannins which are responsible for the bioactivity of the sample fractions. All fractions had a LC_{50} value greater than 1000µg/ml which is an indication that they are all non toxic. Therefore *Indigofera lupatana* Baker F. can be used as an alternative source of new, effective, safe drug(s) against the tested microbes because it demonstrated anti-bacterial efficacy with no toxicity to brine shrimps.

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LIST OF ABBREVIATIONS

AST- Anti-microbial susceptibility tests

ATCC- American type culture collection

BST- Brine shrimp toxicity

CFU- Colony forming units

DMSO- Dimethylsulfoxide

LC- Lethal concentration

LPS- Lipopolysaccharide

MHA- Mueller Hinton agar

MIC- Minimum inhibitory concentration

STD_a- Standard Negative control

STD_b- Standard Positive control

TLC- Thin layer chromatography

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Herbal remedies as cheap alternative to conventional medicine have contributed significantly to rural livelihoods. Apart from the traditional healers practicing herbal medicine, many people are involved in collecting and trading medicinal plants. This has resulted in an increased demand worldwide leading to enhanced new drugs. The World Health Organization (WHO) estimates that 80% of the world's population depends on medicinal plants for their primary health care (Gurib-Fakim and Schmelzer, 2007; Mothana *et al*., 2008).

Natural products are an important source of new anti-microbial agents. Drugs derived from unmodified natural products or semi-synthetic drugs obtained from natural sources accounted for 78% of the new drugs approved by the United States Food and Drug Administration (FDA) between 1983 and 1994 (Suffredini *et al*., 2006). This evidence contributes to the support and quantification of the importance of screening natural products.

Infectious diseases are the world's main cause of human and animal mortality. The situation is further aggravated by the rapid development of multi-drug resistance to available anti-microbial agents (Doughart and Okafor, 2007), their limited anti-microbial spectrum, their side effects (Huie, 2002), and emergence and re-emergence of opportunistic infections. Therefore, studies aimed at finding and characterization of the substances that exhibit activity against infectious micro-organisms, yet showing no cross resistance with existing antibiotics, are urgently required (Olila *et al.,* 2001). In recent years, pharmaceutical companies have focused on developing drugs from natural products. Also, discovery of new drugs has been made a continuous process to counter the limitations of conventional antibiotics (Doughart and Okafor, 2007), and hence the driving force of this study.

Medicinal plants form the largest single group of plants (Gurib-Fakim and Schmelzer, 2007). *Indigofera lupatana* Baker F. locally called '*mugiti'* in Mbeere community of Kenya is a woody shrub found in *Acacia-Combretum* ecological zones of Mbeere District in Kenya. It is widely used for its perceived medicinal value in treating coughs and diarrhea (Riley and Brokensha, 1988), gonorrhea and pleurisy (Kokwaro, 1993). There is apparently no documented scientific report on anti-microbial properties, phytochemical studies and toxicity profiles of this plant. This has often constituted a major constraint to consideration of the use of herbal remedies in conjunction with or as an affordable alternative to conventional medical treatment (Okeke *et al.,* 2001).

The bio-activity of natural products is due to phytochemicals, often elaborated for the plant defense. These phytochemicals inadvertently protect humans against pathogens. Some phytochemicals are known to have therapeutic and prophylactic properties, provide nutrition for normal cell health and repairs, inhibit carcinogens and act as antioxidants. The phytochemical screening of plant materials to determine the presence of bioactive chemical constituents is thus vital in the knowledge of their therapeutic properties (Ogunwenmo *et al.,* 2007). This can be done chemically, or through qualitative thin layer chromatography (TLC).

Knowledge of the chemical constituents of plants is desirable, not only for the discovery of therapeutic agents, but also because such information may be of value in disclosing new sources of such economic substances such as; tannins, oils, gums, and precursors for the synthesis of complex chemical substances. In addition, the knowledge of the chemical constituents of plants would further be valuable in discovering the actual value of folkloric remedies (Mojab *et al.*, 2003).

Despite the beneficial effects of phytochemicals, studies have established that they can be toxic. These chemicals are produced as part of the plant's defense against pests and herbivores or to gain an advantage over competing agents. Therefore, medicinal plants are not always safe (Orech *et al.,* 2005). It is therefore appropriate to evaluate the potential toxicity of the plant extracts (said to indicate useful antineoplastic activity). This

will be used to create awareness of the safety of the plant extract(s) as a medicine (Houghton and Raman, 1998; Orech *et al.*, 2005; Suffredini *et al*., 2006).

1.2 Statement of Problem

Infectious diseases are the world's leading cause of human and animal morbidity and mortality. This is compounded by rapid development of multi-drug resistance to available antibiotics coupled with the negative side effects of these antibiotics. Further, synthetic drugs are unaffordable by the majority in developing countries. Therefore, plants provide a cheaper alternative source of medication. However, there is need to ascertain their safety, in addition to phytochemical characterization. This will reveal therapeutic potentials of the plants and also provide the basis for conservation of herbal plants that face extinction.

1.3 Objectives of the Study

1.3.1 Main Objective

To investigate the anti-bacterial potential, toxicity and characterize the active phytochemicals of *Indigofera lupatana* Baker F.

1.3.2 Specific Objectives

1. To determine the anti-bacterial activity of the *I. lupatana* Baker F. root extracts.
2. To screen the toxicity and the phytochemicals, that may be responsible for the anti-bacterial activity and/or toxicity of the *I. lupatana* Baker F. root extracts.

1.4 Hypotheses

1. There is no significant difference in the anti-bacterial activity of the *I. lupatana* Baker F. root extracts and that of the standard anti-bacterial.
2. The *I. lupatana* Baker. F. root extracts are toxic to *artemia salina* leach.

1.5 Justification of the Study

Animal and human morbidity and mortality are mainly caused by infectious diseases. For instance; typhoid fever kills about 600,000 people with another 13-17 million people being affected annually (Brusch and Garvey, 2006). This is further complicated by multi-drug resistance toward most of the conventional antibiotics (Doughart and Okafor, 2007). In addition, the cost of production and procurement of conventional drugs is too high for developing countries. Therefore, some plants may provide a cheap alternative as direct extracts or as semi-synthetic drugs (Houghton and Raman, 1998).

Most 'herbal' medicines are used as crude extracts extracted with water or local alcoholic brew and are justified through grounds of tradition and anecdotal evidence. Nevertheless, they still form the basis for primary health care in 80% of the developing world (Mothana *et al*., 2008). Although their case is judged 'not proven' (often prejudicially) by western science, some cultures, notably in India and China have evolved a philosophy of disease and medicine which is very sophisticated and can explain the use of a particular plant (Houghton and Raman, 1998). However, it is important to undertake anti-microbial activity, toxicity, and phytochemical profiling of the plant extracts for scientific rationalization and standardization of efficacy and safety (Huie, 2002) and hence popularize them as cheap and affordable alternative to orthodox medical treatment and form the basis for their conservation (Okeke *et al.,* 2001). Knowledge of the chemical constituents of plants helps in disclosing new sources of economic materials such as tannins, oils, biofuels gums, detergents and precursors for the synthesis of complex chemical substances. In addition, it helps in discovering the actual value of folkloric remedies (Mojab *et al.*, 2003)

CHAPTER TWO

LITERATURE REVIEW

2.1 *Indigofera*

Indigofera is a genus of about 700 species of flowering plants belonging to the family *Fabaceae.* They occur throughout the tropical and subtropical regions of the world, with a few species reaching the temperate zone in eastern Asia. The species are mostly shrubs, though some are herbaceous, and a few can become small trees up to 5-6 m tall. Most are dry-season or winter deciduous.

Several of them are used to produce the dye indigo and also to alleviate pain. The herbs are generally regarded as having anti-inflammatory activity (Wikipedia, 2010). *Indigofera articulata* is used to cure toothache, *Indigofera suffruticosa* is used to treat inflammations and epilepsy while *Indigofera oblogofera* is used as an anti-inflammatory for insect stings, snakebites, and tissue swellings (Leite *et al*., 2006). A patent was granted for use of *Indigofera arrecta* extract to relieve ulcer pain (Wikipedia, 2010).

2.2 *Indigofera lupatana* Baker F.

These are woody plants that can reach 2.5 m in height. They are not true shrubs since they are deciduous in the dry seasons. The plant has imparipinnate leaves with 5 to 11 leaflets about 20×12 mm in length and breadth. The inflorescences are in the leaf axils and they can be up to 15 cm in length, being densely crowded with tiny pea-like flowers. The flowers are usually pink but can also turn to reddish mauve after pollination.

Members of this species are found in the acacia-combretum ecological zones of Mbeere in Kenya (Riley and Brokensha, 1988), and in Ethiopia, Tanzania, Malawi, Mozambique, and South Africa (Gillet *et al.,* 1971).

They have medicinal value for treating coughs, diarrhea (Riley and Brokensha, 1988), gonorrhea and pleurisy (Kokwaro, 1993). The plant is scientifically classified as; Kingdom: ***Plantae,*** Division: ***Magnoliophyta,*** Class: ***Magnoliopsida,*** Order: ***Fabales,***

Family: ***Fabaceae,*** Subfamily: ***Faboideae,*** Tribe: ***Indigofereae,*** Genus: ***Indigofera,*** and species: ***Indigofera lupatana*** (Gillett *et al.*, 1971; Wikipedia, 2010).

2.3 Phytochemicals

Phytochemicals are non-nutritive plant chemicals that have prophylactic and/or therapeutic properties. Plants produce these chemicals to protect themselves, but they can also protect humans against diseases.

Hippocrates may have prescribed willow tree leaves to abate fever. Salicin, having anti-inflammatory and pain-relieving properties, was originally extracted from the white willow tree and later synthetically produced to become the stable over-the-counter drug, Aspirin (Huie, 2002). Vicristine and taxol are natural products used for cancer chemotherapy (Houghton and Raman, 1998).

Phytochemicals can have complementary and/or overlapping mechanisms of action in the body, including antioxidant effects, modulation of enzyme actions, boosting of the immune system, modulation of hormone metabolism, anti-bacterial and antiviral effect, interference with DNA replication and physical action whereby some phytochemicals bind physically to cell walls thereby preventing the adhesion of pathogens to human cell membranes (Tony, 2008). Some of these phytochemicals are discussed in the following sections.

2.3.1 Alkaloids

An alkaloid is a plant-derived compound that is toxic or physiologically active. Some alkaloids such as isopteropodine, pteropopine have anti-microbial activity by promoting white blood cells to dispose harmful micro-organisms and cell debris (Ogunwenmo *et al.*, 2007). Highly aromatic planar quaternary alkaloids like berberine, piperine and harmane work by intercalating the DNA and cell wall (Cowan, 1999).

Others, by simulating neurotransmitters such as acetylcholine, dopamine and serotonin, they affect central nervous system (CNS) at the synapses.

Alkaloids act as; narcotics, as analgesic and as anti-malaria. Also, as topical anesthetic for ophthalmology, in treating hypertension, neuralgia, rheumatism, motion sickness, and also in extending the life of hormones (Armstrong, 1998).

Alkaloids have antineoplatic activity, for example, indole alkaloids are used in leukemia and Hodgkin's disease chemotherapy. They act by termination and depolymerization of protein microtubules that form the mitotic spindle in cell division. This process helps in terminating the tumor cells from separating or dividing and henceforth resulting to reduction of cancer (Sneden, 2005). Nevertheless, some types are toxic, hallucinative and addictive, and hence the need to profileboththeir activity and toxicity (Victor *et al.*, 2005).

2.3.2 Tannins/ polyphenols

Tannins are astringent, bitter plant polyphenols that either bind, precipitate or shrink proteins. They have physiological role of acting as antioxidants through free radical scavenging activity, chelation of transition metals, inhibition of pro-oxidative enzymes and lipid peroxidation (Vit *et al*., 2008), hence modulating oxidative stress and preventing degenerative diseases. They also inhibit tumor growth by inducing apoptosis (Scalbert *et al*., 2005) and inhibit mutagenecity of carcinogens (Okuda, 2005). They exhibit anti-microbial activity where they act by complexing nucleophilic proteins through hydrogen bonding, covalent bonding, and nonspecific interactions (Cowan, 1999).

The main targets for complexing are adhesins, cell wall proteins and cell membrane proteins, hence inactivating microbial adhesion which is the first step in establishment of infections, and also causing cell wall/membrane disruption (Cowan, 1999; Victor *et al*., 2005). This also inactivates microbial enzymes and cell envelope

transport proteins by processes that may involve reaction with sulfhydryl groups of proteins.

Phenols also form complexes with metal ions (e.g. cobalt, manganese, iron, copper, etc.) necessary for microbial growth as co-factors and activators of enzymes. They also inhibit viral reverse transcriptase (Ogunwenmo *et al*., 2007).

Toxicity to micro-organisms in phenolic compounds depends on the site and the number of hydroxyl groups, with evidence that increased hydroxylation results to increased toxicity (Cowan, 1999).

Polyphenols have endocrine role by interacting with estrogen receptors. They are also anti-inflammatory, molluscicidal and hence important in the control of schistosomiasis. They also have anti-diarrheal, anti-septic, anti-fungal properties, anti-parasitic, astrigent properties and also used in curbing hemorrhage, in wound healing, and improving vascular health by suppressing peptides that harden arteries (Victor *et al*., 2005).

Tannins have economic role of tanning leathers in leather industry. Nevertheless they affect intake and digestibility of feeds among livestock, and excess can be carcinogenic (Scalbert *et al*., 2005).

2.3.3 Flavonoids

They are structural derivatives of flavones containing conjugated aromatic systems often bound to sugar(s) as glycosides and are water soluble (Harborne, 1973). They exert their roles as anti-oxidants, hence protecting against degenerative disease. Flavonoids such as quercetin, act as chain breaking anti-oxidants and they prevent oxidation of low-density lipoprotein by macrophages and metal ions like copper. This is important in reducing the oxidative stress (Buhler and Miranda, 2000).

Flavonoids also act as ‘nature’s biological modifiers’ as anti-allergens, anti-inflammatory, and induces phase two enzymes that eliminate mutagens and carcinogens

(Buhler and Miranda, 2000; Ogunwenmo *et al*., 2007). They also act as anti-microbial by complexing extracellular proteins, soluble proteins and bacteria cell wall. More lipophilic flavonoids may also disrupt microbial membranes (Al-Bayati and Al-Mola, 2008). Probable targets on microbial cell are surface-exposed adhesins, cell wall polypeptides, and membrane bound enzymes. While others like catechins found in oolong green tea inactivates bacterial toxins (e.g. cholera toxin) and inhibits bacterial glucosyltransferases.

Flavonoids are also known to increase coronary flow, reduce the myocardial oxygen consumption and to lower the arterial pressure (Dong et al., 2005). They are also known to reduce capillary fragility (Harborne, 1973), to be anti-allergic and also to be anti-spasmodic hence applied to relief asthma and nose bleeding (Victor *et al*., 2005). Flavonoids lacking hydroxyl groups (-OH) on their structure are more active against the micro-organism than those having -OH, and this supports the idea that their anti-microbial mechanism may be targeting the membrane (Cowan, 1999).

2.3.4 Saponins

These are survice active agents with soap-like properties and can be detected by their ability to cause foaming and to haemolyse blood cells (Harborne, 1973). They have a host of biological roles which include boosting respiratory system as expectorant, and hence activity against cough. They also have anti-protozoa activity by reacting with cholesterol in the protozoal cell membranes causing cell lysis, for example, Yucca saponins are effective against protozoan *Giardia lamblia* (Cheeke, 1998).

Saponins are used as vaccine boosters by acting as adjuvant, as anti-inflammatory, emetics, anti-viral, anti-fungal, insecticidal, molluscicidal, piscidal and as anti-bacterial by inhibiting colonization and boosting the immunity. The mode of action for the anti-bacterial effects may also involve membranolytic properties of the saponins as well as lowering of the surface tension of the extracellular medium (Al-Bayati and Al-Mola, 2008).

Saponins have antineoplastic activity without killing normal cells. This is by reacting with cholesterol rich membranes of cancer cells, and inducing mitotic arrest that causes apoptosis of cell (Sahelian, 2008). This limits cell division, growth and also binds to primary bile acids, which are metabolized by colon bacteria into secondary bile acids. Some of the secondary bile acids are promoters of colon cancer.

Some saponins like Radix Notoginseng have been reported to increase the blood flow of the coronary arteries, prevent platelet aggregation and to decrease the consumption of oxygen by heart muscles (Dong *et al*., 2005). They also have anti-edema, anti-tussive, purgative and immuno-regulatory properties (Victor *et al*., 2005). Saponins are source of cheap, environment friendly detergents, and cosmetics (Cheeke, 1998).

2.3.5 Phytosteroids

Some phytosteroids are plant steroids that act as weak hormones in the body. They share a common basic ring structure with animal steroids though they are not equivalent because of varying chemical groups attached to the main ring in different positions (Hobbs, 2005). They are used to treat reproductive complications such as venereal diseases. They are also used during pregnancy to ensure an easy delivery, as well as to promote fertility in women and libido in men. They also act as sex hormones derivatives, (for example, they can be metabolized to either androgen or estrogen-like substances) (Victor *et al*., 2005), and hence they are potential source of contraceptives.

Phytosteroids are anti-microbial, analgesic, anti-inflammatory, and are used in treating stomach ailments and in decreasing serum cholesterol levels (Cyberlipid, 2008). They also have been indicated as potent inhibitors of macrophage activation, blocking the production of pro-inflammatory cytokines and LPS-induced lethality and have potential use as immunosuppressive agents (Soares *et al*., 2005).

2.3.6 Terpenoids

These are derivatives of isoprene molecule having a carbon skeleton built from one or more of C_{15} units (Harborne, 1973). They exert their roles as anti-bacteria, anti-fungi, anti-viral, anti-protozoan, anti-allergens, as immune boosters and as antineoplastic agents (Roberts, 2007).

The mechanism of action of terpenoids is speculated to involve membrane disruption by these lipophilic compounds. Petalostemumal has demonstrated activity against *B. subtilis, S. aureus, and C. albicans* and to a lesser extent to Gram-negative bacteria (Cowan, 1999). This may be due to a perturbation of the lipid fraction of bacterial plasma membranes, resulting in alterations of membrane permeability and in leakage of intracellular materials. This is related to physicochemical characteristics of the active principle (such as lipophilicity and water solubility), lipid composition and net surface charge of the bacterial membranes. These phytochemicals can cross the cell membranes, penetrating the interior of the cell and interacting with intracellular targets critical for antibacterial activity (Trombetta *et al.*, 2005).

Terpenoids are used to alleviate epilepsy, to relieve cold, influenza, cough and acute bronchial disease (Victor *et al.*, 2005). From laboratory studies of terpenes, it has been suggested that the possible target of these compounds involves hypothalamus-pituitary-adrenal axis due to the observed effects on the levels of adrenocorticotropic hormone and corticosterone (Briskin, 2000).

2.3.7 Cardiac Glycosides

Cardiac glycosides (also called cardenoloids) occur as a complex mixture together in the same plant. Most of them are toxic, however many have pharmacological activity especially to the heart (Harborne, 1973). They are used in treatment of congestive heart failure, whereby they inhibit Na^+/K^+-ATPase pump that causes positive ionotropic effects and electrophysiological changes. This strengthens heart muscle and the power of systolic concentration against congestive heart failure (Ogunwenmo *et al.*, 2007). They are also

used in treatment of atrial fibrillation, flatter, and they act as emetics and as diuretics (Harborne, 1973; Desai, 2000).

Generally, phytochemicals use several mechanisms of actions to mediate their action, most of which mimics the mechanisms used by conventional antimicrobials.

2.4 Mechanisms of Action of Antimicrobials

Microbial cells grow and divide to reach the large numbers present during an infection. To grow and divide, organisms must synthesize or take up many types of biomolecules. Anti-microbial agents interfere with specific processes that are essential for growth and/or division (Neu and Gootz, 2001). Some mechanisms of action for antibiotics include the following.

(i) Inhibition of Cell Wall Synthesis

This is either through; inhibition of cell wall biosynthetic enzymes (such as by Fosfomycin), drugs combining with carrier molecules (as by Bacitracin), drugs that combine with cell wall substrates (such as by Vancomycin), or drugs that inhibit polymerization and attachment of new peptidoglycan (for example, Penicillin).

(ii) Inhibition of Cytoplasmic Membranes

This is either through; disorganization of cytoplasmic membranes (such as by Polymyxins), making pores in membranes (as by Gramicidins) or disruption of fungi cell membranes (such as by Nystatin).

(iii) Inhibition of Nucleic Acid Synthesis

This is either through; blocking of DNA gyrase which is involved in DNA super coiling (as by Quinolones), blocking mRNA synthesis (as by Rifampin), intercalating

DNA template (as by Lucanthone), or inhibiting nucleotide metabolism (as by Sulfonamide).

(iv) Inhibition of Ribosome Function

This is through inhibition of either the 50S ribosomal sub-unit (such as by Chloramphenicol), or 30S ribosomal sub-units (as by Aminoglycosides) (Neu and Gootz, 2001).

2.5 Anti-Microbial Resistance

Pathogenic microbes cause infectious diseases, and anti-microbial agents have been developed to combat the spread and severity of many of these diseases. The use of an anti-microbial for any infection, in any dose and over any time period, forces microbes to either adapt or die under "selective pressure".

The microbes which adapt and survive carry genes for resistance (WHO, 2002). Therefore, antibiotic resistance results from gene action, encoded on plasmids or on the chromosome. Bacteria acquire genes conferring resistance by process of spontaneous DNA mutations, conjugation, and transposition (Lewis, 1995). The mechanisms of resistance include;

(i) Altering the Target of the Drug

Beta-lactam drugs like penicillin and cephalosporin induces synthesis of new penicillin binding protein which does not bind β-lactam drugs due to diminished affinity. This form of resistance is exhibited by *S. aureus and N. gonorrhea*. Sulfonamide resistance is due to altered or new dihydropteroic synthetase that has poor affinity for sulfonamides.

Therefore, alteration of the target reduces its physiological importance, and this leads to synthesis of a new enzyme that duplicates the functions of the inhibited target, or makes the target insensitive to the drug (Neu and Gootz, 2001).

(ii) Prevention of Access of the Drug to the Target

This is through efflux of more drug(s) from the target site than the one getting concentrated there (for example, efflux pump of tetracycline drug have been demonstrated in *S. aureus, Pseudomonas* species and *Salmonella* species) (Brusch and Garvey, 2006). It also involves modification of the drug so that it fails to enter the cell (e.g. aminoglycosides gets phosphorylated, adenylated and acetylated) hence it cannot traverse membranes to get concentrated at target sites (Brusch and Garvey, 2006).

(iii) Inactivation of the Drug Agent

This involves destruction or modification of the drug agent by microbe enzymes so that it fails to bind to the target. For example, β-lactam resistance is due to exoenzyme β-lactamase elaborated by bacteria *E. coli*, *S. aureus*, *Salmonella* species, *Pseudomonas* species *and Klebsiella* species that destroys the β-lactam ring (Neu and Gootz, 2001). Chloramphenicol resistance is due to transacetylase enzyme that acetylate hydroxyl group of Chloramphenicol so that it binds less to 50S ribosome subunit (Neu and Gootz, 2001).

The effects of drug resistance are treatment failure leading to prolonged illness, risk of death, and longer periods of infectivity, which increase the population of 'carriers' in the community and thus expose the general population to the risk of contracting a resistant strain of infection. When infections become resistant to first-generation anti-microbials, treatment has to be switched to second- or third-generation drugs, which are more expensive and more toxic (WHO, 2002). Therefore, conventional medicine is increasingly receptive to use of plants as an alternative source of drugs. This is because plants' active principles have novel modes of actions that show no cross resistance with

conventional antibiotics (Houghton and Raman, 1998). They also have low toxicity potential (Cowan, 1999).

2.6 Crude Extract Fractionation Procedures

Components of a mixture, such as an extract from plant can be separated into groups of compounds sharing similar physico-chemical characteristics. Separation is achieved due to particular features in compounds, such as solubility, size, shape, polarity, electrical charge and others. Common methods of fractionation based on these features includes; precipitation, solvent extraction, distillation, dialysis, electrophoresis and chromatographic procedures (Houghton and Raman, 1998).

2.6.1 Solvent Extraction

This method involves bringing the material to be extracted (usually in solid form) into contact with the extraction solvent for a period of time, followed by separation of the solution from the solid debris. Fractionation is achieved by use of solvents of different polarity, as each solvent will extract different phytochemical class(es) e.g. Hexane will extract waxes, fats and volatile oils, Chloroform and Dichloromethane will extract alkaloids, aglycones and volatile oils, Ethyl acetate and Acetone will extract alkaloids, aglycones and glycosides while Methanol will extract sugars, amino acids and glycosides. Solvent extraction methods include percolation, infusion, reflux extraction and sohlet extraction (Houghton and Raman, 1998).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Collection and Identification of Plant Samples

The plant samples for the study were collected from Mbeere district, in the Eastern province of Kenya. The plant was taxonomically authenticated at the Department of Biological Sciences of Egerton University. A voucher sample was assigned a reference number (NSN1) and banked in the same department herbarium.

3.2 Plant Root Preparation and Extraction

The plant roots were separated, washed, cut into small pieces, air-dried in the dark to avoid decomposition of light sensitive bio active compounds (Houghton and Raman, 1998), at room temperature to a constant weight and ground into a powder by a mill (Thomas-Wiley laboratory mill, model 4). The powder was extracted by sequential process of four organic solvents of increasing polarity; hexane, ethyl acetate, dichloromethane, and methanol in that order (Houghton and Raman, 1998).

Ground material (150 g) was soaked in the solvent, starting with the less polar (hexane) for 24 hours at room temperature with intermittent shaking followed by decanting and filtration by gravity to separate the debris. Fresh solvent was replaced and agitated for 10 minutes, decanted and filtered. The two volumes were combined together and concentrated in rotary vacuum evaporator (BÜCHI ROTAVAPOR R-205 V805, Flawil, Switzerland) and allowed to air dry. The process was repeated with residue agitated in Ethyl acetate, Dichloromethane and finally in Methanol in the same manner as above (Houghton and Raman, 1998; Wojcikowski *et al.*, 2008).

3.3 Collection of Test Micro-organisms

A total of eight standard bacterial strains were used which were maintained on agar slant at 4 °C in the microbiology laboratory in Biochemistry department of Egerton University. They were: three Gram-positive bacterial species (*Bacillus subtilis* BGA spores suspension which were ready-to-use (Merck, Darmstadt, Federal Republic of Germany), *Bacillus cereus* ATCC 11778 spores which were ready-to-use preparation (Difco Laboratories, Detroit, Michigan, USA) and S*taphylococcus aureus* ATCC 25923 (KEMRI) and five Gram-negative bacteria (*Escherichia coli* ATCC 25922 (KEMRI), *Pseudomonas aeruginosa* ATCC 27853 (KEMRI), *Salmonella typhimurium* ATCC 13311 (KEMRI), *Klebsiella pneumoniae* and *Proteus mirabilis* (clinical isolates from KEMRI). *Artemia salina* leach were used for brine shrimp lethality bioassay. They were to be artificially hatched from eggs sourced from commercial aquariums.

3.4 Anti-microbial tests

The tests guide on the choice of appropriate agents for therapy, provide a range of suitable alternatives and accumulated data from which information on the most suitable agents for empirical use can be derived. They are also used to evaluate *in vitro* activity of the anti-microbial agents. The results are either reported qualitatively as Sensitive, Intermediate, or Resistant or quantitatively in terms of MIC and MBC) (Collins *et al*., 1995).

3.4.1 Culture media

Nutrient agar was used for sub-culturing of the test micro-organisms, at 37^0C for 24 hrs and the Mueller Hinton agar was used for sensitivity assay (Nguemeving *et al.,* 2006).

3.4.2 Standards

Chloramphenicol was used as a standard drug for positive control (STD_b) against bacteria. Its choice was based on its properties as a broad spectrum drug, a very stable drug under a variety of conditions of temperature and humidity, and its low toxicity threshold when ingested (Drew *et al*., 1972). The aqueous 1% Dimethylsulfoxide (DMSO) was used as solvent for the extracted samples because it is amphipathic, able to diffuse well in the agar and at this concentration it is non toxic (Moshi *et al*., 2006; Mbaveng *et al.,* 2008). Therefore, aqueous 1% DMSO was used as negative control (STD_a).

3.4.3 Anti-bacterial susceptibility tests

(a) Media preparation

The media was reconstituted using distilled water according to the manufacturer's instructions, sterilized by autoclaving at 121^0C and pressure of 15 psi for 15 minutes. It was then dispensed aseptically into Petri dishes (9 cm diameter), a volume of between 18-25 ml molten agar to achieve a depth of between 3-4 mm, and left to solidify and then stored in the refrigerator at 4^0C. The inoculation plates were air dried with the lids ajar until there were no moisture droplets on the petri dish surfaces (Collins *et al.,* 1995).

(b) Preparation of discs

Whatmann filter paper (No. 1) discs of 6 mm diameter were made by punching the paper, and the blank discs were sterilized in the hot air oven at 160^0C for one hour. They were then impregnated with 10 µl of the varying concentration of extract fraction solution. Each fraction stock solution (hexane fraction (1.5 g/ml), ethyl acetate fraction (1.4 g/ml), dichloromethane fraction (1.0 g/ml) and methanol (1.6 g/ml)) was serially diluted at two folds. The discs were dried by heating at 50^0C (Ayo *et al*., 2007). The

STD_b (chloramphenicol at 30 µg/disc) was used as positive controls and a disc loaded with 10 µl of aqueous 1% DMSO used as a negative control (STD_a) (Mbwambo *et al.,* 2007; Mbaveng *et al.,* 2008).

(c) Disc diffusion test

The anti-bacterial activity was assayed by disc diffusion method according to Ayo *et al*., (2007), CLSI (2007) and Mbaveng *et al.,* (2008). The bacterial strains were activated by growing them in nutrient agar at 37^0C for 18 to 24 hours. A fresh inoculum was developed by suspending activated colonies in physiological saline solution (0.85% NaCl). An inoculum of bacterial cell suspension of about 1.5×10^6 CFU/ml was determined and standardized using a McFarland turbidity standard No. 0.5. The suspension was authenticated by adjusting the optical density to 0.1 at 600 nm.

This suspension was used to aseptically inoculate by swapping the surface of MHA plates. Excess liquid was air-dried under a sterile hood. The impregnated discs were then planted at equidistant points on top of the inoculated agar medium by sterile forceps. A disc prepared with only the corresponding volume of aqueous 1% DMSO was used as a negative control, while chloramphenicol was used as positive control. The inoculated plates were incubated at 4^0C for 2 hours to allow the pre-diffusion of extracts into the media. The plates were then incubated at 37^0C for 24 hrs. Anti-bacterial activity was evaluated by measuring the diameter of the inhibition zone. The lowest concentration of the extract that inhibited bacterial growth was recorded as the MIC of the extract fraction (Mothana *et al*., 2008).

3.5 Phytochemical Tests

Phytochemical tests were done to determine the class of compounds in the active fractions. These were identified by characteristic colour changes using standard

procedures according to Houghton and Raman, (1998), Edeoga *et al*., (2005), and Shanmugavalli *et al.,* (2009), and the results were reported as (+ve) for presence, and (-ve) for absence. They included the following tests:

3.5.1. Alkaloids

A portion of extract (0.2 g) was dissolved in 1 ml of 1% sulphuric acid. To the acid solution, a drop of mayer's reagent was added and a white to buff precipitate would show presence of alkaloids. (Houghton and Raman, 1998; Shanmugavalli *et al.,* 2009).

3.5.2. Saponins

About 0.2 g of powdered sample extract was boiled in 2 ml of distilled water on a water bath and filtered. A fraction of aqueous filtrate measuring 1 ml was mixed with 2 ml of distilled water and shaken vigorously to form a stable persistent froth. The frothing was mixed with a about three drops of olive oil and shaken vigorously. Formation of an emulsion confirmed presence of saponins (Edeoga *et al.,* 2005).

3.5.3. Tannins

About 0.2 g of the dried powdered samples were boiled in 10 ml of distilled water in a test tube and then filtered. Positive tests were confirmed by addition of 0.1% $FeCl_3$ solution, resulting in a characteristic blue, blue-black, green or blue-green color (Edeoga *et al.,* 2005)

3.5.4. Test for phlobatannins

About 2 g of powdered sample was boiled with 1% aqueous hydrochloric acid for 5 minutes. A positive test result was confirmed by deposition of a red precipitate (Edeoga *et al.,* 2005).

3.5.5. Test for flavonoids

About 5 ml of dilute aqueous ammonia solution was added to a portion of the aqueous filtrate of the plant extract, followed by addition of concentrated sulphuric acid. A positive test result was confirmed by the formation of a yellow coloration that disappeared instantly (Edeoga *et al.,* 2005).

3.5.6. Test for steroids

About 2 ml each of acetic anhydride and sulphuric acid was added to about 0.5 g of solvent extract. A positive test result was confirmed by change of colour from violet to blue or green (Edeoga *et al.,* 2005; Shanmugavalli *et al.,* 2009).

3.5.7. Test for terpenoids (Salkowski test)

About 5 ml of the extract was mixed with 2 ml of chloroform and 3 ml of concentrated H_2SO_4 was added to form a layer. A positive test result was confirmed by presence of a reddish brown colouration at the interface (Edeoga *et al.,* 2005).

3.5.8. Test for cardiac glycosides (Keller-Killiani test)

About 5 ml of the extract was mixed with 2 ml of glacial acetic acid containing one drop ferric chloride solution. To this, 1 ml of concentrated sulphuric acid was slowly underlayed to the sample mixture. A positive test result was confirmed by the presence of a brown ring at the interface (Edeoga *et al.*, 2005).

3.6 Chemical group tests

The fractions were tested for some of the functional groups as follows:

3.6.1. Phenolic group test

To test for the presence of phenolic groups, 3 to 5 drops of 1M $NaOH_{(aq)}$ were added to 2 ml of the sample. Solubility of the sample was an indication of presence of phenolic groups (Pavia, 1990).

3.6.2. Carboxylic acid group test

To test for the presence of carboxylic acid groups, 3-5 drops of 1M $NaHCO_{3\,(aq)}$ were added to 2 ml of sample extract. Solubility and effervescence of the sample was a confirmation of a presence of carboxylic groups (Pavia, 1990).

3.6.3. Lucas test for alcohol groups

To test for the presence of alcohol/hydroxyl groups, 3-5 drops of Lucas reagent were added to 2 ml of the sample. Formation of green precipitate was a confirmation of positive results. If the reaction took place very fast, tertiary alcohols were present, if moderate reaction took place, secondary alcohol were present and where reaction was unobservable, it indicated presence of primary alcohols (Jerry *et al.,* 1998).

3.6.4. Potassium permanganate test for unsaturation or hydroxyl group

To test for the presence of double and/or triple bonds or OH groups, 3-5 drops of 1 M Potassium permanganate was added drop wise and shaken. Decolourization of potassium permanganate was a confirmation of a positive test (Furniss *et al.,* 1989).

3.6.5. Tollen's test for aldehyde and/or ketone groups

To test for the presence of aldehydes and/or ketones, 3-5 drops of Tollen's reagent were added to 2 ml of the sample. Presence of silver or a black precipitate was a confirmation of a positive test (Jerry *et al.,* 1998).

3.7 Brine shrimp toxicity bioassay

The brine shrimp (*Artemia salina* leach) toxicity bioassay was conducted according to Orech *et al.,* (2005) and Ayo *et al.,* (2007). Artificial seawater was prepared by dissolving sea salt (38.0 g) in distilled water (1 L). The seawater was put in a small tank and a teaspoon of brine shrimp eggs was added to one side of the divided tank, which was covered. The other side was not covered so as to allow light that attracted the hatched shrimps. The tank containing the brine shrimp eggs was left at room temperature for 48 hours to allow the eggs to hatch. Different concentrations of plant extract fractions were prepared, using 1% aqueous DMSO. This involved dissolving sample extract (30 mg) in DMSO (3 ml). From this solution, concentrations (1000, 500, 250, 125, and 62.5 µg/ml) were obtained respectively by serial dilution. An aliquot of each concentration (1 ml) was transferred, in triplicates, into clean sterile universal vials with pipette and artificial sea water (9 ml) was added. Ten shrimp nauplii were added to each vial (30 shrimps per concentration). DMSO aqueous solution (1%) was used as the negative control. All test tubes were incubated at room temperature for 24 hrs. After this period, the number of the dead and the surviving brine shrimps was recorded, and percentage death at each concentration was determined. The LC_{50} value at 95% confidence interval

was determined from the counts using the probit analysis. The criterion for toxicity for fractions was established as LC_{50} value > 1000 µg/ml (non toxic), ≥500≤1000 µg/ml (weak toxicity) and <500 µg/ml (toxic) (Orech *et al.*, 2005; Ayo *et al.*, 2007; Mbwambo *et al.*, 2007; Bastos *et al.*, 2009).

3.8 Statistical analysis

The hypothesis 1 was analysed using paired *t*-test to compare the anti-bacterial activity of the sample fractions with the standard antibiotic and test values expressed as mean ± standard deviation. $P < 0.05$ value was considered to indicate statistically significant difference between the test sample and the standard. The hypothesis 2 was tested with Probit Analysis using the EPA computer probit analysis program (Version 1.5).

CHAPTER FOUR

RESULTS AND DISCUSSION

RESULTS

4.1 Phytochemical results for *Indigofera. lupatana* Baker F. roots extracts

The phytochemical screening of the extracts of *Indigofera lupatana* Baker F. revealed that alkaloids were absent in all extract fractions while phytosteroids were present in all sample fractions (Table 1). Flavonoids, saponins, cardiac glycosides and terpenoids were absent in hexane extracts but all were present in ethyl acetate, dichloromethane and methanol extract fractions. Tannins were present in the more polar extracts of methanol and dichloromethane but absent in the ethyl acetate and non-polar hexane fraction. Phlobatannins were present in the hexane, ethyl acetate and methanol fractions, but absent in the dichloromethane extract fraction

Table 1: Phytochemical tests results.

Phytochemical constituent	**Hexane extract**	**Ethyl acetate extract**	**Dichloromethane extract**	**Methanol extract**
Alkaloids	-ve	-ve	-ve	-ve
Flavonoids	-ve	+ve	+ve	+ve
Tannins	-ve	-ve	+ve	+ve
Saponins	-ve	+ve	+ve	+ve
Cardiac glycosides	-ve	+ve	+ve	+ve
Phlobatannins	+ve	+ve	-ve	+ve
Phytosteroids	+ve	+ve	+ve	+ve
Terpenoids	-ve	+ve	+ve	+ve

(+ve) - Represent presence of the tested phytochemicals in the sample fraction

(−ve) - Represent absence of the tested phytochemicals in the sample fraction

4.2 The Chemical results for *Indigofera lupatana* Baker F. roots extracts

The chemical tests revealed presence of phenolic groups, carboxyl groups and hydroxyl groups in ethyl acetate, dichloromethane and methanol extract fractions, but they were absence in the hexane fraction. Aldehyde and/or ketone compounds were however present in all extract fractions while unsaturated compounds were present in all fractions except dichloromethane extract fraction.

Table 2: Chemical tests result.

Chemical constituent	**Hexane extract**	**Ethyl acetate extract**	**Dichloromethane extract**	**Methanol extract**
NaOH test for Phenolic Group	-ve	+ve	+ve	+ve
$NaHCO_3$ test for Carboxyl Group	-ve	+ve	+ve	+ve
Lucas Test for Hydroxyl Groups	-ve	+ve	+ve	+ve
$KMnO_4$ Test for unsaturation	+ve	+ve	-ve	+ve
Tollen's Test for Aldehyde and/or Ketone Groups	+ve	+ve	+ve	+ve

(+ve) - Represent presence of the tested chemical groups in the sample fraction

(−ve) - Represent absence of the tested chemical groups in the sample fraction

4.3 Anti-bacterial activity result for the root extracts of *Indigofera lupatana* Baker F.

The anti-bacterial activity test revealed extracts activity to both Gram positive and Gram negative bacteria. The activity varied depending with the extract fraction tested, extract concentration and the test bacteria.

4.3.1 Hexane fraction

The general inhibition by hexane extract fraction was low with the highest inhibition giving a zone of 6.5±0.5 mm and MIC of 750 mg/ml. Gram positive bacteria and *P. aeruginosa* were not sensitive to hexane extract fraction while *E. coli, K. pneumoniae, P. mirabilis and S. typhimurium* all had inhibition zones of 6.5±0.3, 6.0±0, 6.3±0.3 and 6.3±0.3 mm respectively and MIC of 750 mg/ml (Table 3).

Table 3: Anti-bacterial activity result for the hexane root extract fraction.

Micro organism	**Inhibition zones diameter in mm**							**MIC(mg/ml)**	
	Extract concentration (µg x 10^2)					**STD_a**	**STD_b**		
	150	75	37.5	18.8	9.4		**30µg**	**E**	**STD_b**
Gram negative bacteria									
E. coli	6.5 ± 0.3	0	0	0	0	0	48.3±1.7	750	25
K. pneumoniae	6.0±0	0	0	0	0	0	37.6 ± 0.8	750	22.5
P. aeruginosa	0	0	0	0	0	0	24.3 ± 1.3	>750	NT
P. mirabilis	6.3±0.3	0	0	0	0	0	34.3 ± 1.3	750	NT
S. typhimurium	6.3±0.3	0	0	0	0	0	29.0 ± 1.5	750	NT
Gram positive bacteria									
S. aureus	0	0	0	0	0	0	37.3 ± 0.9	>750	31.3

STD_a –Represent 1% DMSO as negative control; STD_b – Represents Chloramphenicol as positive control; E – Represents extract fraction and NT – Represent not tested. Values are inhibition zones in mm (mean ± SEM; n = 3).

4.3.2 Ethyl acetate fraction

The fraction showed marked activity to all bacteria tested. Gram positive bacteria had inhibition zones ranging from 7.0±0.1, 21.2 ± 0.6 and 26.0 ± 0.3mm and MIC of 700, 43.8 and 21.9 mg/ml in *S. aureus, B. subtilis and B. cereus* respectively. However, Gram negative bacteria had inhibition zones of 16.3 ± 0.7, 10.6 ± 0.9, 21.5 ± 0.9, 10.4 ± 1.1 and 17.3 ± 0.3mm and MIC of 175, 350, 175, 350 and 700 mg/ml in *E. coli, K. pneumoniae, P. aeruginosa, P. mirabilis and S. typhimurium* respectively (Table 4).

Table 4: Anti-bacterial activity result for the ethyl acetate root extract.

Micro organism	**Inhibition zones diameter in mm**							**MIC(mg/ml)**	
	Extract concentration (µg x 10^2)					**STD_a**	**STD_b**		
	140	**70**	**35**	**17.5**	**8.8**		**30µg**	**E**	**STD_b**
Gram negative bacteria									
E. coli	16.3 ± 0.7	14.0±1.0	12.0±0.5	0	0	0	48.3 ± 1.0	175	25
K. pneumoniae	10.6 ± 0.9	8.0±0.5	0	0	0	0	37.6 ± 0.8	350	22.5
P. aeruginosa	21.5 ± 0.9	10.0±1.0	7.0±0	0	0	0	24.3 ± 1.3	175	NT
P. mirabilis	10.4 ± 1.1	6.0±0	0	0	0	0	34.3 ± 1.3	350	NT
S. typhimurium	17.3 ± 0.3	0	0	0	0	0	29.0 ± 1.5	700	NT
Gram positive bacteria									
S. aureus	7.0±0.1	0	0	0	0	0	37.3 ± 0.9	700	31.3
B. cereus	21.2 ± 0.6	19.5±0.5	15.0±1.0	13.5±0.5	8.0±1.7	0	22.3 ± 0.8	43.8	NT
B. subtilis	26.0 ± 0.3	24.5±0.5	23.0±1.0	21.0±0.6	18±0.5	0	32.6 ± 0.9	21.9	26.3

STD_a –Represent 1% DMSO as negative control; STD_b – Represents Chloramphenicol as positive control; E – Represents extract fraction and NT – Represent not tested. Values are inhibition zones in mm (mean ± SEM; n = 3).

4.3.3 Dichloromethane fraction

Dichloromethane fraction had inhibition zones of 22.0 ± 0.1, 28.5 ± 0.3 and 22.6 ± 1.0mm and MIC of 62.5, 250 and 31.3 mg/ml among Gram positive *B. cereus, S. aureus and B. subtilis* bacteria respectively. Gram negative bacteria gave inhibitions of 21.7 ± 0.7, 11.4 ± 0.8, 8.3 ± 0.6, 10.7 ± 0.7 and 8.3 ± 0.6mm and MIC of 125, 250, 125, 125 and 500 mg/ml in *E. coli, K. pneumoniae, P. aeruginosa, P. mirabilis and S. typhimurium* respectively (Table 5).

Table 5: Anti-bacterial activity result for the dichloromethane root extract fraction.

Micro organism	**Inhibition zones diameter in mm**							**MIC(mg/ml)**	
	Extract concentration (µg x 10^2)					**STD_a**	**STD_b**		
	100	**50**	**25**	**12.5**	**6.3**		**30µg**	**E**	**STD_b**
Gram negative bacteria									
E. coli	21.7 ± 0.7	14.0±0.6	11.0±1	0	0	0	48.3±1.0	125	25
K. pneumoniae	11.4 ± 0.8	7.7±0.3	0	0	0	0	37.6 ± 0.8	250	22.5
P. aeruginosa	10.0 ± 0.7	8.3±0.3	7.0±0	0	0	0	24.3 ± 1.3	125	NT
P. mirabilis	10.7 ± 0.7	9.2±0.7	7.0±0.5	0	0	0	34.3 ± 1.3	125	NT
S. typhimurium	8.3 ± 0.6	0	0	0	0	0	29.0 ± 1.5	500	NT
Gram positive bacteria									
S. aureus	22.6 ± 1.0	12.4±2.8	0	0	0	0	37.3 ± 0.9	250	31.3
B. cereus	22.0 ± 0.1	20.5±0.5	17.0±2.0	12.0±0	0	0	22.3 ± 0.8	62.5	NT
B. subtilis	28.5 ± 0.3	27.5±0.5	21.5±0.5	19.0±0.6	18±0	0	32.6 ± 0.9	31.3	26.3

STD_a –Represent 1% DMSO as negative control; STD_b – Represents Chloramphenicol as positive control; E – Represents extract fraction and NT – Represent not tested. Values are inhibition zones in mm (mean ± SEM; n = 3).

4.3.4 Methanol fraction

Methanol extract had inhibition zones of 16.7 ± 0.7, 22.0 ± 0.3 and 28.0 ± 0.7 mm and MIC of 400, 25 and 25 mg/ml among Gram positive *S. aureus, B. cereus and B. subtilis* bacteria respectively. Gram negative bacteria had inhibition zones of 21.7 ± 0.5, 15.3 ± 0.4, 11.7 ± 0.8, 11.3 ± 0.7 and 12.3 ± 0.5mm and MIC of 100, 200, 100, 400 and 400 mg/ml among *E. coli, K. pneumoniae, P. aeruginosa, P. mirabilis and S. typhimurium* respectively (Table 6).

Table 6: Anti-bacterial activity result for the methanol root extract fraction.

Micro organism	**Inhibition zones diameter in mm**							**MIC(mg/ml)**	
	Extract concentration (µg x 10^2)					**STD_a**	**STD_b**		
	160	**80**	**40**	**20**	**10**		**30µg**	**E**	**STD_b**
Gram negative bacteria									
E. coli	21.7 ± 0.5	15.5±1.0	13±0.8	10±0.4	0	0	48.3±1.0	100	25
K. pneumoniae	15.3 ± 0.4	9.0±1.2	7.0±0.5	0	0	0	37.6 ± 0.8	200	22.5
P. aeruginosa	11.7 ± 0.8	9.0±0.5	7.0±0	6.0±0	0	0	24.3 ± 1.3	100	NT
P. mirabilis	12.3 ± 0.5	9.0±1	0	0	0	0	34.3 ± 1.3	400	NT
S. typhimurium	11.3 ± 0.7	6.0±0.0	0	0	0	0	29.0 ± 1.5	400	NT
Gram positive bacteria									
S. aureus	16.7 ± 0.7	8.5±0.5	0	0	0	0	37.3 ± 0.9	400	31.3
B. cereus	22.0 ± 0.3	20.6±0.5	20.0±0	16.5±0.5	15.7±0.7	0	22.3 ± 0.8	25	NT
B. subtilis	28.0 ± 0.7	23.0±0.6	21.7±0.7	19.5±0.5	16.0±1.0	0	32.6 ± 0.9	25	26.3

STD_a –Represent 1% DMSO as negative control; STD_b – Represents Chloramphenicol as positive control; E – Represents extract fraction and NT – Represent not tested. Values are inhibition zones in mm (mean ± SEM; n = 3).

Therefore among the Gram positive bacteria, *B. subtilis* was the most sensitive giving the lowest MIC and the highest inhibition zones of 21.9mg/ml and 28.12mm respectively while among the Gram negative bacteria, the most sensitive bacteria was *E. coli* with the highest inhibition of 22.1mm and MIC of 125mg/ml. Generally, Gram positive bacteria were more sensitive to extracts responding with the lowest MIC compared to Gram negative bacteria. The inhibition values for chloramphenicol ranged between 48.3±1.0 and 22.3 ± 0.8mm and MIC of between 31.3 to 22.5 mg/ml in the tested bacteria.

4.4: T-test result for the root extracts of *Indigofera lupatana* Baker F.

The extract fractions of *I. lupatana* Baker F. had anti-bacterial activity against Gram positive and Gram negative bacteria. The negative control had no inhibition on any test bacteria, however all bacteria tested were sensitive to the chloramphenicol positive control.

By comparing the inhibition zones of the extracts with that of chloramphenicol using paired students t-test at $P<0.05$, most of the fractions exhibited significant difference in their inhibitions in respect to the positive control except Ethyl acetate fraction activity to *P. aeruginosa* and *B. cereus*, (P values of 0.3257 and 0.4863 respectively); Dichloromethane fraction toward *B. cereus*, (P values of 0.7462) and Methanol fraction toward *B. cereus*, (P values of 0.8) as shown in Table 7.

Table 7: T-test result for the root extracts of *Indigofera lupatana* Baker F.

Micro organism	Extract Fractions	Inhibition Zones Diameter in mm		P value
		E	STD_b	
Gram negative				
E. coli	Hexane	6.5 ± 0.3	48.3 ± 1	0.0003*
	Ethyl acetate	16.3 ± 0.7	48.3 ± 1	0.0003*
	Dichloromethane	21.7 ± 0.7	48.3 ± 1	0.0036*
	Methanol	21.7 ± 0.5	48.3 ± 1	0.0004*
K. pneumoniae	Hexane	6.0 ± 0	37.6 ± 0.8	0.0007*
	Ethyl acetate	10.6 ± 0.9	37.6 ± 0.8	0.0041*
	Dichloromethane	11.4 ± 0.8	37.6 ± 0.8	0.0035*
	Methanol	15.3 ± 0.4	37.6 ± 0.8	0.0029*
P. aeruginosa	Hexane	0	24.3 ± 1.3	0.003*
	Ethyl acetate	21.5 ± 0.9	24.3 ± 1.3	**0.3257**
	Dichloromethane	10.0 ± 0.7	24.3 ± 1.3	0.0193*
	Methanol	11.7 ± 0.8	24.3 ± 1.3	0.026*
P. mirabilis	Hexane	6.3 ± 0.3	34.3 ± 1.3	0.0014*
	Ethyl acetate	10.4 ± 1.1	34.3 ± 1.3	0.0101*
	Dichloromethane	10.7 ± 0.7	34.3 ± 1.3	0.0012*
	Methanol	12.3 ± 0.5	34.3 ± 1.3	0.0066*
S. typhimurium	Hexane	6.3 ± 0.3	29.0 ± 1.5	0.0031*
	Ethyl acetate	17.3 ± 0.3	29.0 ± 1.5	0.0249*
	Dichloromethane	8.3 ± 0.6	29.0 ± 1.5	0.0023*
	Methanol	11.3 ± 0.7	29.0 ± 1.5	0.0022

Gram positive				
S. aureus	Hexane	0 ± 0	37.3 ± 0.9	0.0005*
	Ethyl acetate	7.0 ± 0.06	37.3 ± 0.9	0.0009*
	Dichloromethane	22.6 ± 1.0	37.3 ± 0.9	0.0001*
	Methanol	16.7 ± 0.7	37.3 ± 0.9	< 0.0001*
B. cereus	Ethyl acetate	21.2 ± 0.6	22.3 ± 0.8	**0.4863**
	Dichloromethane	22.0 ± 0.1	22.3 ± 0.8	**0.7462**
	Methanol	22.0 ± 0.3	22.3 ± 0.8	**0.8**
B. subtilis	Ethyl acetate	26.0 ± 0.3	32.6 ± 0.9	0.02914
	Dichloromethane	28.5 ± 0.3	32.6 ± 0.9	0.0192
	Methanol	28.0 ± 0.7	32.6 ± 0.9	0.0016

E – Represents extract fraction

STD_b – Represents positive control

Values for E and STD_b are given as mean ± SEM

$P < 0.05$

*- Represent significance difference between STD_b and E

4.5 Toxicity test result for the Root Extracts of *Indigofera lupatana* Baker F.

The brine shrimp lethality bioassay results were determined using mortality data. From the probit analyses, it was established that all extract fractions had a LC_{50} value greater than 1000µg/ml and hence none was toxic (Table 8).

Table 8: Toxicity test result after 24 hours

Extract fraction	**LC_{50} (μg/ml)**	**95% CI**	**Slope (± SE)**	**χ^2**
Hexane	1370.78	974.76 – 3514.65	2.88 ± 0.80	1.05
Ethyl acetate	2335.15	2026.33 – 2737.86	4.97 ± 0.66	4.61
Dichloromethane	1858.39	1589.06 – 2221.05	3.69 ± 0.47	4.27
Methanol	1248.09	1087.95 – 1457.43	3.87 ± 0.46	3.70

LC_{50}- means the concentration that kills 50% of the population (in μg/ml); 95% confidence interval gives the fiducial limit; 1% DMSO solution was used as negative control and caused no mortality to brine shrimps; CI – Represent confidence interval.

DISCUSSION

Arrays of phytochemicals were detected in the sample fractions. These phytochemicals included: flavonoids, tannins, saponins, cardiac glycosides, steroids, phlobatannins and terpenoids. They are normally produced by plants as an evolutionary adaptation to harsh environment or in response to attack by other organisms (Ogunwenmo *et al.,* 2007). They however have been found to inadvertently confer anti-microbial protections to humans due to compounds synthesized in the secondary metabolism (Samy and Gopalakrishnakone, 2008) as well as being immuno-modulative (Okuda., 2005; Al-Bayati and Al-Mola, 2008).

Tannins were detected in dichloromethane and methanol extracts only. They have physiological role by acting as antioxidants through free radical scavenging activity, chelation of transition metals, inhibition of pro-oxidative enzymes and lipid peroxidation (Navarro *et al.,* 2003; Vit *et al.,* 2008), hence modulating oxidative stress and preventing degenerative diseases. They also inhibit tumor growth by inducing apoptosis (Scalbert *et al.,* 2005) and inhibit mutagenecity of carcinogens (Okuda., 2005). They exhibit anti-microbial activity by complexing proteins such as adhesins, substrates and cell membrane proteins, hence inactivating microbial adhesion which is the first step in establishment of infections, and also causing membrane disruption (Cowan, 1999; Okuda, 2005; Victor *et al.,* 2005; Biradar *et al.,* 2007). They also inactivate microbial enzymes and cell envelope transport proteins by processes that may involve reaction with sulfhydryl groups or through non-specific interaction with the proteins (Samy and Gopalakrishnakone, 2008; Kaur and Arora, 2009).

Phenolics were also detected in ethyl acetate, dichloromethane and methanol extracts fractions. They function by complexing metal ions (e.g. cobalt, manganese, iron, copper, etc.) necessary for microbial growth as co-factors and activators of enzymes (Okuda, 2005; Biradar *et al.,* 2007). They also have anti-viral activity by inhibiting viral reverse transcriptase and inducing DNA fragmentation, and they potentiate host-immune defense (Okuda, 2005; Biradar *et al.,* 2007; Ogunwenmo *et al.,* 2007). Toxicity to micro-organisms in phenolic compounds depends on the site and the number of hydroxyl

groups, with evidence that increased hydroxylation results to increased toxicity (Przybylski *et al.,* 1998; Cowan, 1999; Biradar *et al.,* 2007; Samy and Gopalakrishnakone, 2008). This was justified further by detection of hydroxyl groups in these extract fractions (Table 2).

Both tannins and phenolics have endocrine role, and they function by interacting with estrogen receptors (Victor *et al.,* 2005). They are also anti-inflammatory, molluscicidal and hence important in the control of schistosomiasis (Victor *et al.,* 2005). They also have anti-diarrheal, anti-septic, anti-viral, anti-fungal, anti-parasitic, anti-irritant properties, used in curbing hemorrhage, in wound healing, and improving vascular health by suppressing peptides that harden arteries (Victor *et al.,* 2005; Awoyinka *et al.,* 2007; Ogunwenmo *et al.,* 2007).

Flavonoids were also detected in all fractions except hexane fractions. They exert their roles as chain breaking anti-oxidants, and by preventing oxidation of low-density lipoprotein by macrophages and metal ions like copper. This reduces the oxidative stress (Buhler and Miranda, 2000). They also act as 'nature's biological modifiers' as anti-allergens, anti-inflammatory, and induces phase two enzymes that eliminate mutagens and carcinogens (Ogunwenmo *et al.,* 2007). They also act as anti-microbial by complexing extracellular and soluble proteins, and bacteria cell wall. More lipophilic flavonoids may also disrupt microbial membranes (Navarro *et al.,* 2003; Al-Bayati and Al-Mola, 2008; Samy and Gopalakrishnakone, 2008; Kaur and Arora, 2009). Probable targets on microbial cell are surface-exposed adhesins, cell wall polypeptides, and membrane bound enzymes. Still, they may inactivate bacterial toxins (e.g. cholera toxin) and inhibits bacterial glucosyltransferases. Flavonoids are also known to increase coronary flow, to reduce the myocardial oxygen consumption and to lower the arterial pressure (Dong *et al*., 2005). They are also known to reduce capillary fragility (Harborne, 1973), to be anti-trypanocidal (Navarro *et al.*, 2003), anti-allergic and also to be anti-spasmodic and hence applied to relief asthma and nose bleeding (Victor *et al.,* 2005). Flavonoids lacking hydroxyl groups (-OH) on their structure are more active against the

micro-organism than those having -OH, and this supports the idea that their microbial target is the membrane (Cowan, 1999; Samy and Gopalakrishnakone, 2008).

Saponins were also detected in all fractions except hexane fractions. They boost respiratory system as expectorant, and hence activity against cough. This could perhaps justify the already traditionally established function of the plant in the treatment and management of dry coughs. They also have anti-protozoa activity by reacting with cholesterol in the protozoal cell membranes causing cell lyses (Cheeke, 1998). Also, saponins functions as vaccine adjuvant, as anti-inflammatory, emetics, anti-viral, antifungal, insecticidal, molluscicidal, piscidal and as anti-bacterial by inhibiting colonization and boosting the immunity. The mode of action for the anti-bacterial effects may involve membranolytic properties of the saponins as well as lowering of the surface tension of the extracellular medium (Al-Bayati and Al-Mola, 2008). They have antineoplastic activity where they act by reacting with cholesterol rich membranes of cancer cells, and inducing mitotic arrest that causes apoptosis of cell (Sahelian, 2008). This limits cell division and growth. They also bind to primary bile acids, which are metabolized by colon bacteria into secondary bile acids. Some of these are promoters of colon cancer (Cheeke, 1998). Also, saponins increase the blood flow of the coronary arteries, prevent platelet aggregation and decrease the consumption of oxygen by heart muscles (Dong *et al.*, 2005). They also have anti-edema, anti-tussive, purgative, anti-hypercholesterol, hypotensive, cardiac depressant and immuno-regulatory properties (Victor *et al.*, 2005; Awoyinka *et al.*, 2007).

Terpenoids were also detected in all fractions except hexane fraction. They exert their roles as anti-bacteria, anti-amoebic, anti-fungi, anti-viral, anti-protozoan, anti-allergens, as immune boosters and as antineoplasia (Ogunwenmo *et al.*, 2007; Roberts, 2007). The mechanism of action is speculated to involve membrane disruption by these lipophilic compounds (Cowan, 1999; Ogunwenmo *et al.*, 2007; Samy and Gopalakrishnakone, 2008). This may involve perturbation of the lipid fraction of bacterial plasma membranes, altering membrane permeability hence causing leakage of intracellular materials. This is related to physicochemical characteristics of the active

principle such as lipophilicity and water solubility, lipid composition and net surface charge of the bacterial membranes. These phytochemicals can cross the cell membranes, penetrating the interior of the cell and interacting with intracellular targets critical for antibacterial activity (Trombetta *et al.*, 2005). They are also used to alleviate epilepsy, to relieve cold, influenza, cough and acute bronchial disease (Victor *et al.*, 2005), and this could offer a justification why the plant is used in managing cough.

From (Table 4, 5 and 6), ethyl acetate, dichloromethane and methanol extracts showed anti-microbial activity toward most of the tested bacteria, and this could be due to presence of saponins, flavonoids, phenolics and terpenoids that were detected in these fractions.

Phytosteroids and cardiac glycosides were also detected in plant extract fractions. Phytosteroids are used to treat venereal diseases, used in pregnancy to ensure an easy delivery and hormonal balance as well as to promote fertility in women and libido in men. They also act as starting material in the synthesis of sex hormones (Edeoga *et al.*, 2005; Victor *et al.*, 2005) and hence they are potential source of contraceptives. They are also anti-microbial, analgesic, anti-inflammatory, and immuno-suppressive by inhibiting macrophage activation, blocking the production of pro-inflammatory cytokines. They are also active in managing stomach ailments and in decreasing serum cholesterol levels (Soares *et al.*, 2005).

Cardiac glycosides are used in treatment of congestive heart failure, whereby they inhibit Na^+/K^+-ATPase pump that causes positive ionotropic effects and electrophysiological changes. This strengthens heart muscle and the power of systolic concentration against congestive heart failure (Ogunwenmo *et al.*, 2007). They are also used in treatment of atrial fibrillation, flatter, and they acts as emetics and as diuretics (Harborne, 1973; Desai, 2000; Awoyinka *et al.*, 2007).

Phlobatannins were also found to be present in all extracts fractions except dichloromethane fraction, and their presence suggests the diuretic property (Awoyinka *et al.*, 2007) of the plant.

However alkaloids were absent in all the extract fractions, and this could offer justification for the non-toxicity of the plant extracts, since most of alkaloids are associated with toxicity (Sneden, 2005; Victor *et al.,* 2005; Ogunwenmo *et al.,* 2007).

The plant extracts had broad spectrum activity in that they inhibited growth of both Gram positive and Gram negative bacteria. The inhibition zones increased on increasing the concentration of the extract in the discs showing a concentration dependent activity and also varied with the species of bacteria tested. Although the concentrations of the extract fractions were in the range of 100 times more than the standard antibiotic (chloramphenicol), they showed marked anti-bacterial activity as evidenced by their zones of inhibition. This could be due to the fact that the active components in the extract comprise only a fraction of the extract used. Therefore, the concentration of the active components in the extract could be much lower than the standard antibiotic used. It is important to note that, if the active components were isolated and purified, they would probably show higher antibacterial activity than those observed in this study.

The hexane fraction had the lowest sensitivity to the test bacteria. The extract fraction showed the highest inhibition zone of 6.5±0.5 mm in *E. coli and S. typhimurium* and showed no inhibition to *P. aeruginosa* and *S. aureus.* This low activity could be due to absence of phytochemicals such as flavonoids, tannins, saponins, cardiac glycosides, terpenoids and phenolics that have been associated with antimicrobial activity or due to antagonistic actions of specific compounds in the hexane crude extract (Kokwaro, 1993; Cowan, 1999; Ogunwenmo *et al.,* 2007; Al-Bayati and Al-Mola, 2008).

Ethyl acetate fraction showed marked sensitivity to the tested bacteria. In *P. aeruginosa, B. cereus and B. subtilis*, there was no significant difference ($p<0.05$) in the inhibition zones of the extract fraction and the inhibition zones of chloramphenicol standard (Table 7). The highest sensitivity was recorded in *B. subtilis* at inhibition zone of 26±0 mm and MIC of 21.9 mg/ml. This antibacterial activity could be due to flavonoids that have been shown to act by complexing proteins and disrupting membranes (Navarro *et al.,* 2003; Al-Bayati and Al-Mola, 2008; Samy and Gopalakrishnakone, 2008; Kaur and Arora, 2009), saponins that have been demonstrated

to act by inhibiting bacterial colonization, lowering surface tension of extracellular medium or by lysing bacterial membranes (Al-Bayati and Al-Mola, 2008), phytosteroids and terpenoids that acts by disrupting bacterial membrane (Cowan, 1999; Soares *et al.*, 2005; Ogunwenmo *et al.*, 2007; Samy and Gopalakrishnakone, 2008). Cardiac glycosides and phlobatannins that were also detected could be responsible for antibacterial activity.

The dichloromethane fraction showed marked activity in all tested bacteria, with highest inhibition of 28.5mm and lowest MIC of 31.3 mg/ml in *B. subtilis.* There was no statistically significant difference at ($p< 0.05$) between the extracts inhibition zones and zones of inhibition of the chloramphenicol standard. This activity was due to phytochemicals detected in the extract fraction such as tannins that acts by complexing bacterial proteins, interfering with bacterial adhesion, inactivating enzymes and disrupting bacterial cell membrane (Cowan, 1999; Okuda, 2005; Victor *et al.*, 2005; Biradar *et al.*, 2007; Samy and Gopalakrishnakone, 2008; Kaur and Arora, 2009). Also, activity could be due to flavonoids, saponins, cardiac glycosides, phytosteroids and terpenoids that were detected in the extract and have been shown to have antibacterial activity (Kokwaro, 1993).

The methanol fraction yielded highest overall inhibition of 28.0mm in *B. subtilis* and the lowest MIC of 25 mg/ml in both *B. subtilis* and *B. cereus.* The activity was broad spectrum and could be due phytochemicals tested in this fraction. Methanol extract tested positive to all phytochemicals except alkaloids (Table 1). Although traditional healers make use of water as herbal solvent, studies have shown that methanol solvent is much better and powerful (Wojcikowski *et al.*, 2007). This could be due to the polarity of the solvent that conferred the ability to extract a variety of compounds and could be the justification for the reasons why methanol extracts shown better inhibitions (Parekh and Chanda, 2006). Polarity of the solvent also influences the qualitative and quantitative composition of the active compounds in extract fractions, hence the differences in the bioactivities of the various extract fractions (Houghton and Raman, 1998; Doughari and Okafor, 2007; Wojcikowski *et al.*, 2007; Tomczykowa *et al.*, 2008).

The bioactivities demonstrated by the different types of extract fractions may be attributed to the diversity of structures and/or the uneven distributions of chemical constituents within these extract fractions. Each extract had a different degree of inhibitory activity and specificity against bacteria and this is related to the polarity of the extracting solvent (Przybylski *et al.,* 1998). The polar solvents extracted phenolics, tannins, flavonoids, terpenoids that are responsible for the bioactivity of these extracts and could either have exhibited synergistic or additive effects when used in their crude form (Mohamed *et al.,* 2010).

Gram positive strains were more susceptible to the extract than Gram negative strains. This is in agreement with previous reports that plant extracts are more active against Gram positive bacteria than Gram negative bacteria (Parekh and Chanda, 2006; Mohamed *et al.,* 2010). The higher sensitivity of Gram-positive bacteria could be attributed to their outer peptidoglycan layer which is not an effective permeability barrier as compared to the outer phospholipid membranes of Gram-negative bacteria (Trombetta *et al.,* 2005; Tomczykowa *et al.,* 2008; Kaur and Arora, 2009).

The present work showed the potential of tested extract against the causative agents of nosocomial infections and morbidity among immuno compromised and severely ill patients such as *P. aeruginosa, S. aureus* (Bastos *et al*., 2009; Kaur and Arora, 2009). Infections caused by *P. aeruginosa* and *B. cereus* are difficult to combat (Aliero. and Afolayan, 2005) and therefore their susceptibility to the extracts which showed no statistical difference ($P<0.05$) in their inhibition zones as compared to the inhibitions of the same bacteria by the chloramphenicol (Table 7) is a pointer to extracts potential as a drug against these bacteria.

The plant extracts also showed commendable activity toward pathogen responsible for the gastrointestinal disorders that leads to diarrhea, coleocystitis, and urinary tract infections e.g. *E. coli, S. typhimurium* (Moshi *et al.,* 2006; Matasyoh *et al*., 2007) and this supports the traditional use of this plant for the treatment of diarrhea (Riley and Brokensha, 1988).

The brine shrimp (*Artemia salina* leach) are used in the laboratory bioassay of toxicity through estimation of medium lethal concentration (LC_{50}) (Lieberman, 1999; Ayo *et al.*, 2007). Several studies have established BST as an excellent benchtop, simple bioassay for the preliminary investigations in discovery, purification, isolation and research of natural products (Lieberman, 1999). The technique is a low-cost test, easily mastered bioassay, utilizing small amount of the test material (Bastos *et al.*, 2009), and it has been used for preliminary assessment of anti-bacterial, toxicity, pesticidal, antineoplastic and insecticidal activity (Suffredini *et al.*, 2006), detecting fungal toxins, cynobacteria toxins, heavy metals, food additives, cytotoxic testing of dental materials, home cleaning products and pharmaceuticals (Carballo *et al*., 2002; Lieberman, 1999). Furthermore, studies have showed that there is a positive correlation between the lethality to brine shrimp and corresponding oral lethal dose, and therefore the bioassay present a useful alternative model for predicting the oral acute toxicity of plant extract as well as a model for bioassay-guided fractionation of active toxic and antitumor agent (Parra *et al.*, 2001; Ayo *et al.*, 2007; Bastos *et al.*, 2009).

According to Parra *et al*. (2001), Navarro *et al*. (2003) and Bastos *et al*. (2009), LC_{50} values less than 1000µg/ml is considered toxic. All tested extract fractions had a LC_{50} value greater than 1000µg/ml (Table 8) and therefore all the plant extracts were not toxic.

This finding is important in support of the use of this plant for alternative medication. Although members of Mbeere communities have used this plant for centuries without raising any issue of its toxicity, this experiment provide a scientific justification on the safety and selectivity of the bioactive compounds in this plant.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1 Conclusions

From this study the following conclusions were made:

- Anti-bacterial testing showed that *Indigofera lupatana* Baker F. extract fractions had broad spectrum bioactivity as they inhibited both Gram-positive and Gram-negative bacteria. This supports the traditional usage of this plant for therapeutic purposes.
- The extracts demonstrated better activity to Gram-positive bacteria than to Gram negative bacteria.
- Activity of the ethyl acetate, dichloromethane and methanol extracts on *bacillus cereus* species and that of ethyl acetate on *P. aeruginosa* was similar to the activity of chloramphenicol drug (with $P > 0.05$). This demonstrated that at this concentration the extracts can be used as an alternative drug. Since the activity was shown to be dose dependent, better inhibition against other bacteria could be attained by increasing the extract concentration.
- Phytochemical testing showed that the extracts are rich in tannins, saponins, terpenoids, flavonoids, phenolics, phlobatannins, Phytosteroids and cardiac glycosides. These compounds are responsible for the anti-bacterial activities of the assayed fractions, and therefore reflect a potential for the development of novel chemotherapeutic agents or templates which in future may serve as leads for the production of synthetically improved therapeutic agents.
- Brine shrimp toxicity test showed that all the extract fractions were non-toxic (with values of LC50 >1000μg/ml) justifying the safety of the plant against toxicity.
- This validated information should be useful to traditional healers and patients on judicious use of the *Indigofera lupatana* Baker F. plant, as its efficacy and

safety can to some extent be guaranteed having demonstrated broad spectrum anti-microbial activity with no toxicity.

- This *in vitro* study demonstrated that folk medicine can be as effective as modern medicine in combating pathogenic micro organisms. The millenarian use of these plants in folk medicine suggests that they represent an economical and safe alternative to treat multi drug resistant, emerging and re-emerging infectious diseases.

5.2 Recommendations

- More detailed investigations at molecular level should be undertaken to unveil the exact mechanisms of action and reveal the drug targets in microorganisms tested.
- The most active fractions should be subjected to isolation, identification, characterization, purification and elucidation of structures of individual bioactive compounds and carry out further their pharmacological investigations.
- There is need to investigate into possible drug interactions of *Indigofera lupatana* Baker F. bioactive components with conventional anti-microbials, to forestall possible treatment failure or resistance.

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APPENDICES

Appendix 1: A photograph of *Indigofera* lupatana Baker F. (General outlook).

Appendix 2: A photograph of *Indigofera lupatana* Baker F. (Floral parts)