Evaluation of Antimicrobial Activity and Toxicity of Crude Extracts from Selected Kenyan Medicinal Plants

Lilian Cherotich Ngeny

A thesis submitted in partial fulfillment for the degree of Master of Science in Biochemistry in the Jomo Kenyatta University of Agriculture and Technology

2012

DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

Signature…… Date…….

Lilian Cherotich Ngeny

This thesis has been submitted for examination with our approval as university supervisors.

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This work is dedicated to my parents, Timothy Ngeny and Nancy Ngeny, my husband Caleb Nyangate, and siblings, Peter Mutai, Japheth Mutai, Mercy Ngeny, Robert Mutai and Sheila Ngeny. Thank you for being there for me.

ACKNOWLEDGEMENTS

I wish to express my sincerest appreciation to my academic supervisors, Prof. Esther Magiri, Dr. Christine Bii and Dr. Charles Mutai for supervising this research and for invaluable advice and guidance during the entire time of my study.

I would also like to extend my appreciation to staff at Center for Traditional Medicine and Drug Research, especially Nicholas Mwikwabe, Dr. Festus Tolo, Dr. Lucia Keter and Simon Mwangi for guidance, assistance and input during my time in the laboratory. I also would like to sincerely thank Mr. Richard Korir from Center for Microbiology Research, KEMRI, for the assistance he offered during the antimicrobial studies.

Finally I acknowledge the contribution of my parents, siblings and my husband for urging me on and supporting me through the entire period. I will always be grateful to God for you. God Bless you.

I would also want to thank the International Foundation for Science (IFS), for the research grant awarded to Dr. Christine Bii for funding the laboratory work.

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ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
DCM	Dichloromethane
DMSO	Dimethyl Sulfoxide
HEX	Hexane
HIV	Human Immunodeficiency Virus
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KEMRI	Kenya Medical Research Institute
METH	Methanol
MHA	Meuller Hinton Agar
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin Resistant *Staphylococcus aureus*
SDA	Sabourand Dextrose Agar
TLC	Thin Layer Chromatography

ABSTRACT

The world health organization (WHO) estimates that 80% of population in Africa relies on traditional remedies for their healthcare, with very few studies carried out to establish the therapeutic effects of these remedies. From literature search, four medicinal plants; *Asparagus racemosus* (Willd.) (Asparagaceae/Liliaceae), *Ekebergia capensis* Sparrm. (Meliaceae), *Fuerstia africana* T.C.E. Fries (Labiatae) and *Hagenia abyssinica* (Bruce) J.F.Gmel (Rosaceae) were investigated with the overall aim of determining their antimicrobial activity and toxicities. Plants were collected and extracted with organic and aqueous solvents. Antimicrobial activity was determined using the disc diffusion assay technique. Cytotoxicity studies using Vero E6 cell lines and acute toxicity in mice was also determined. In the results, hexane and dichloromethane extracts of leaves *H. abyssinica* at 1000 µg/disc, had significant antibacterial activity against *Staphylococcus aureus* and methicillin resistant *S. aureus* (MRSA). These extracts also displayed low minimum inhibitory concentrations (MIC) ranging from < 1.95 µg/disc to 31.25 µg/disc. However, the plants studied had weak antifungal activity. Hexane and dichloromethane extracts of leaves of *H. abyssinica* were found to be cytotoxic with IC_{50} of < 8 µg/ml. These extracts were tested for acute toxicity and found to be safe at 5000 mg/bwt. The results of the study support the medicinal use of these plants and indicate that useful compounds from *H. abyssinica* can be isolated for further exploitation.

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND INFORMATION

The Human Immunodeficiency Virus (HIV) that causes AIDS is the most important public health problem in Sub-Saharan Africa. An estimated 33.3 million people are living with HIV worldwide, with 22.5 million of them found in Sub-Saharan Africa (UNAIDS, 2010). The estimated number of people living with HIV in Kenya is between 1.3 million and 1.6 million (UNGASS, 2010).

The Human Immunodeficiency Virus acts by depleting CD4 cells in the human body. This therefore weakens the immune system of the host. WHO clinical HIV staging 2 – 4 are characterized by bacterial and fungal infections (WHO, 2007). This accounts for 70% of HIV/AIDS defining illnesses. Opportunistic infections associated with HIV include recurrent upper respiratory tract infections (bacteria sinusitis), oral and vulvovaginal candidiasis, pulmonary tuberculosis pneumonia, non-typhoid salmonella among others (Buwa and van Staden, 2006).

Management of opportunistic infections is through use of antibiotics such as Penicillin and Chloramphenical, and antifungal agents like Fluconazole and Nystatin.

Nevertheless, the draw back in their use is the emerging resistance and associated toxicity of some drugs in use (Jabra- Rizk, 2006).

Microbes that are resistant to first- line drugs are threatening the gains made by the discovery of antimicrobial drugs. Bacterial infections that contribute the most to human disease are also those in which microbial resistance is most evident. Examples include Penicillin resistant *Streptococcus pneumoniae*, Vancomycin resistant *enterococci*, Methicillin resistant *Staphylococcus aureus* and multi drug resistant *Mycobacterium tuberculosis*. As a result, treatment has to be switched to second or third line drugs that are always much more expensive and sometimes more toxic (UNAIDS, 1998).

Since ancient times, plants have provided mankind with a broad and structurally diverse array of pharmacologically active compounds that continue to be utilized as highly effective drugs to combat a multitude of deadly diseases or as lead structures for the development of novel synthetically derived drugs that mirror their models from nature (Jachak and Saklani, 2007).

Traditionally due to their easy accessibility, terrestrial plants and microorganisms have proven to be the richest sources of medicinally useful natural products that are

indispensible in treatment of diseases of public health importance such as malaria and cancer (Proksch *et al*., 2002). Examples of plant derived drugs include Quinine, Artemisinin, and Etoposide.

About 25% of all pharmaceutical sales are drugs derived from plant natural products and an additional 12% are based on microbial produced natural products such as adramycin and bleomycin from various *Streptomyces* strains (Rajeev and Xu, 2004).

Plants with medicinal properties continue to receive attention as scientist survey plants of ethno-botanical significance for biological activities ranging from antimicrobial properties to antitumor properties. A number of plants have been documented to be used as antimicrobial agents in folkloric practises (Kokwaro, 1993; Beentje, 1994; Gachathi, 1989). Based on their traditional uses and ethno botanical information, four plants were selected from two districts in Rift valley province. The plants were *Hagenia abyssinica* (Bruce) J.F. Gmel, *Fuerstia africana* T.C.E. Fries, *Ekebergia capensis* Sparrm and *Asparagus racemosus* (Willd.).

1.2 PROBLEM STATEMENT

Opportunistic microbial infections cause high morbidity and mortality among many patients. Antimicrobial agents currently in use have many limitations such as unwanted side effects, rapid development of resistance and high cost. Thus there is need to search for new antimicrobial agents which can overcome the challenges faced by current conventional drugs. This can be done through screening of traditional medicinal plants used by various communities to determine their biological activity against such infectious agents. Those found to be active can be further investigated to determine their active principles, and therefore act as candidates for new drugs.

1.3 JUSTIFICATION OF THE STUDY

The number of immunocompromised patients in the world has significantly increased due to cancer chemotherapy, organ or bone marrow transplantations and the human immunodeficiency virus (HIV) among others. These immunocompromised people are susceptible to opportunistic infections caused by both bacteria and fungi. The cost of imported conventional drugs is quite high, and some of them have been reported to be toxic to patients and ineffective to drug resistant isolates of some strains of opportunistic microbes. Screening of medicinal plants for antimicrobial activity would possibly result in discovery of plant species with potential herbal antimicrobial agents that can be developed for use against microbial infections.

1.4 RESEARCH QUESTIONS

i. Do the selected medicinal plants reported to treat microbial infections locally have antimicrobial properties?

ii. Do the selected medicinal plants have overt toxicity?

1.5 HYPOTHESIS

Crude extracts of *Hagenia abyssinica*, *Fuerstia africana*, *Asparagus racemosus* and *Ekebergia capensis* are non-toxic with antimicrobial activities.

1.6 STUDY OBJECTIVES

1.6.1 General objective

To determine antimicrobial activity and toxicity of extracts from four selected Kenyan medicinal plants.

1.6.2 Specific objectives

1. To collect, authenticate and prepare extracts from selected medicinal plants.
2. To determine *in vitro* antibacterial and antifungal activities of extracts of selected Kenyan medicinal plants.
3. To evaluate *in vitro* cytotoxicity of active extracts against VERO cell lines.
4. To evaluate *in vivo* acute toxicity of active extracts using Swiss mice.
5. To determine presence of phytochemical compounds on the active extracts.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 OPPORTUNISTIC MICROBIAL INFECTIONS

An opportunistic infection is an infection caused by pathogens (bacterial, viral, fungal or protozoan) that usually do not cause disease in a healthy host. They occur frequently in immunodeficient patients with majority arising from the Human Immunodefiency Virus (HIV) pandemic. Currently there are 33.3 million people living with HIV globally, of who 22.5 million are living in Sub- Saharan Africa (UNAIDS, 2010).

As immunosuppression from HIV infection progresses, the susceptibility and incidences of opportunistic infections increases. Some of the bacterial opportunistic infections include tuberculosis (TB, caused by *Mycobacterium tuberculosis*), bacterial pneumonia and septicemia, bacterial sinusitis, *Mycobacterium avium* complex disease, while opportunistic fungal infections include candidiasis, cryptococcosis, penicilliosis, aspergillosis among others(UNAIDS, 1998).

Other causes of immunosuppression include: aggressive cancer chemotherapy, use of immunosuppressive drugs for organ transplant recipients, use of prosthetic devices, antibiotic use among others (Zhang *et al*., 2007).

2.1.1 Opportunistic bacterial infections

2.1.1.1 Non- typhoid salmonellosis (NTS)

Non-typhoidal *Salmonella* are important food borne pathogens that cause gastroenteritis, bacteremia, and subsequent focal infection. These pathogenic bacteria are especially problematic in a wide variety of immunocompromised individuals, including (but not limited to) patients with malignancy, human immunodeficiency virus, or diabetes, and those receiving corticosteroid therapy or treatment with other immunotherapy agents (Hohmann, 2001). In recent years, NTS bacteraemia has been increasingly reported as a cause of life-threatening infection in immunocompromised hosts. Malignancy and HIV/AIDS are the two most common conditions associated with NTS bacteraemia.

2.1.1.2 Bacterial pneumonia

Pseudomonas aeruginosa is a significant opportunistic human pathogen that causes bacterial pneumonia. Other infections associated with *P. aeruginosa* include urinary tract infections (UTI) and bacteremia. Immunocompromised patients such as HIV/AIDS, cancer and transplant patients are particularly susceptible (Cowan, 1999).

2.1.1.3 Shigellosis

It's a disease caused by *shigella* species of bacteria such as *Shigella flexneri*. The infections are common among HIV patients and can lead to either mild or severe cases of shigellosis. People infected with the bacteria release it into their stool. The bacteria can spread from an infected person to contaminate water or food, or directly to another person (Kristjansson *et al*., 1994).

2.1.2 Opportunistic fungal infections

2.1.2.1 Candidiasis

Also known as 'thrush', candidiasis refers to fungal infections caused by any *Candida* spp. of which *Candida albicans* is the most common. *Candida* being a normal flora in practically all humans, it has many opportunities to cause endogenous infections in compromised hosts. Hence *Candida* infections are the most frequent opportunistic fungal infections (Walsh and Dixon, 1996).

The two main types of candidiasis are localized diseases of the mouth, throat and vagina, (Oral thrush and vaginitis) and systemic disease of the esophagus and disseminated disease (UNAIDS, 1998). *Candida* infections of the latter category are

also referred to as candidemia and are usually confined to severely immunocompromised persons, such as cancer, transplant, and AIDS patients

More than 95% of *Candida* associated blood stream infections are caused by five major species: *Candida albicans, Candida glabrata, Candida parapsilosis, Candida tropicalis* and *Candida krusei* (Pei-Lan *et al*., 2007)

2.1.2.2 Aspergillosis

This is a group of diseases caused by *Aspergillus* spp. *Aspergillus* spp. occurs worldwide and lives in soils and plant debris. Unlike invasive candidiasis, invasive aspergillosis occurs predominantly in highly immunocompromised patients. The most common pathogenic species are *Aspergillus fumigatus* and *Aspergillus flavus. Aspergillus fumigatus* accounts for the majority of cases of invasive aspergillosis (Pei-Lan *et al*., 2007).

Invasive aspergillosis has emerged as a major cause of life-threatening infections in immunocompromised patients. Patients with prolonged neutropenia, inherited immunodeficiency, or those receiving immunosuppressive agents are at risk of infection (Walsh *et al.*, 2008)

2.1.2.3 Cryptococcosis

Cryptococcosis is an infection caused by *Cryptococcus neoformans*. It's a systemic mycose causing 5% of all HIV- associated deaths worldwide. Cryptococcosis often appears as meningitis, and occasionally as pulmonary or disseminated disease. Cryptococcal meningitis (CRM) is the most frequent systemic fungal infection in HIV-infected persons. Without treatment, cryprococcosis can lead to death (UNAIDS, 1998).

2.2 ANTIMICROBIAL DRUGS

An anti-microbial is a substance that kills or inhibits the growth of bacteria, fungi, or protozoans (Aarestrup, 2006). Only drugs that affect the pathogenic microorganisms more adversely than the host (selective toxicity) are administered systemically to humans. The mechanism of action of drugs accounts for its degree of selective toxicity. The most selective toxic antimicrobial agents are those affecting microbial structures (e.g., cell wall) and metabolic pathways (e.g., folic acid biosynthesis) that are not present in eukaryotic cells. On the contrary, antimicrobial agents acting on DNA are more likely to induce toxic effects (Aarestrup, 2006).

2.2.1 Types of antibiotics

Antibiotics are generally agents used to treat bacterial infections with minimum toxicity to humans and other animals. Antibacterial agents differ in both chemical structure and mode of action. Drugs that kill bacteria are defined as bactericidal while those that inhibit or delay growth of bacteria as bacteriostatic. The type of activity of the drug is dependent on how the drug binds to its target. Bactericidal drugs bind irreversibly or with high affinity to the target, whereas bacteriostatic drugs form weak bonds (Neu, 1992). The main classes of antibiotics include; Beta- lactams, Macrolides, Fluoroquinolones, Sulfonamides, Tetracyclines and Aminoglycosides.

2.2.1.1 Beta-lactams

These include Penicillins and Cephalosporins. They work by interfering with inter-peptide linking of peptidoglycan inhibiting synthesis of the peptidoglycan layer of bacterial cell wall. β-lactam antibiotics are analogues of D-alanyl-D-alanine - the terminal amino acid residues on the precursor NAM/NAG-peptide subunits of the nascent peptidoglycan layer. The structural similarity between β-lactam antibiotics and D-alanyl-D-alanine facilitates their binding to the active site of penicillin-binding proteins (PBPs). The β-lactam nucleus of the molecule irreversibly binds to (acylates) the Ser_{403} residue of the PBP active site. This irreversible inhibition of the PBPs

prevents the final crosslinking (transpeptidation) of the nascent peptidoglycan layer, disrupting cell wall synthesis.

Under normal circumstances, peptidoglycan precursors signal a reorganisation of the bacterial cell wall and, as a consequence, trigger the activation of autolytic cell wall hydrolases. Inhibition of cross-linkage by β-lactams causes a build-up of peptidoglycan precursors, which triggers the digestion of existing peptidoglycan by autolytic hydrolases without the production of new peptidoglycan. As a result, the bactericidal action of β-lactam antibiotics is further enhanced. Examples include, Cephalexin, Amoxicillin (Neu, 1992; Van Bambeke, 1999).

2.2.1.2 Macrolides

It's a clinically important family of antibiotics and includes Erythromycin and clarithromycin. They are commonly used to treat gram positive bacteria. They are bacteriostatic and inhibit protein synthesis in bacteria. They bind reversibly to the23S rRNA molecule in the 50S subunit of bacterial ribosomes in the vicinity of the peptidyl transferase center blocking the exit of the growing peptide, thus arresting cell growth (Figure 2.1). The association between macrolides and the ribosome is reversible and

takes place only when the 50S subunit is free from tRNA molecules bearing nascent peptide chains (Gaynor and Mankin, 2003; Ophardt, 2003).

2.2.1.3 Fluoroquinolones

These are broad spectrum bactericidal antibiotics and include Ciprofloxacin, Norfloxacin and Trovafloxacin. They inhibit DNA synthesis in several ways in bacteria causing rapid cell death. The quinolones target bacterial topoisomerases, specifically DNA gyrase (also termed topoisomerase type II) and topoisomerase IV. Quinolones bind the DNA-DNA gyrase (topoisomerase II) complex blocking further DNA replication. Quinolones also block topoisomerase IV, interfering with separation of interlocked replicated DNA molecules (Walker, 1999).

2.2.1.4 Sulfonamides

These are broad spectrum antibiotics and include Cotrimoxazole. They inhibit folic acid production in bacteria leading to cell death. The target of sulfonamides, and the basis for their selectivity, is the enzyme dihydropteroate synthase (DHPS) in the folic acid biosynthetic pathway. Mammalian cells are not dependent on endogenous synthesis of folic acid and generally lack DHPS. Instead, they have a folate uptake system which most prokaryotes lack (Skold, 2000).

2.2.1.5 Tetracyclines

These are protein synthesis inhibitors and include Tetracycline and Doxycycline. They are bacteriostatic in nature. They inhibit bacterial protein synthesis by preventing aminoacyl- tRNA molecules from binding to the mRNA- ribosome complex. They do so mainly by binding reversibly to the 30S ribosomal subunit in the mRNA translation complex, arresting cell growth by inhibiting translation (Figure 2.1) (Chopra and Roberts, 2001).

2.2.1.6 Aminoglycosides

This class of antibiotics includes Gentamicin and Kanamycin. They interfere with protein synthesis by binding to bacterial 30S ribosomal subunit causing misreading of the genetic code, thereby inhibiting translocation (Figure 2.1) (Davies and Wright, 1997).

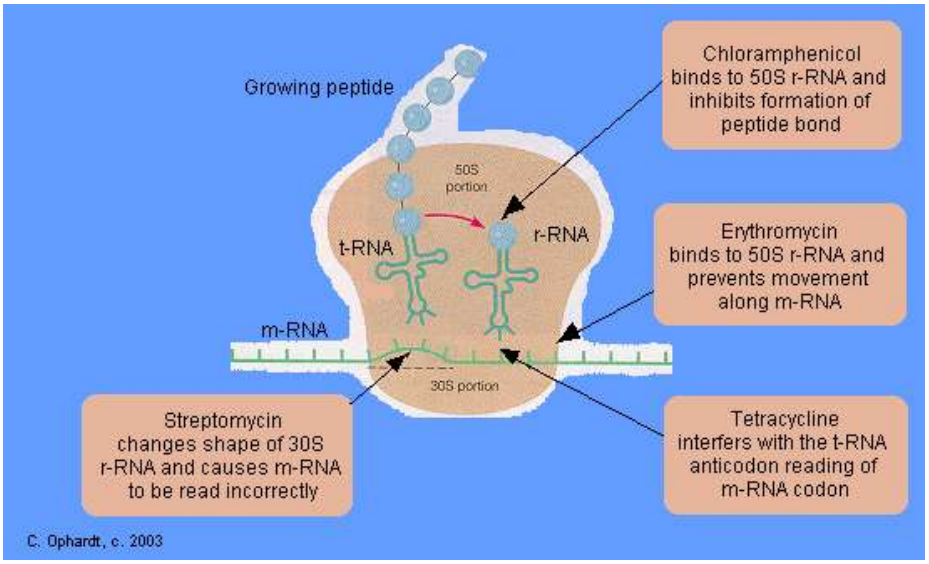


Figure 2. 1: Inhibition of Protein synthesis by antibiotics.

2.2.2 Types of antifungal agents

An antifungal drug is medication used to treat fungal infections such as athlete's foot, ringworm, candidiasis (thrush), serious systemic infections such as cryptococcal meningitis, and other systemic mycoses.

Antifungals work by exploiting differences between mammalian and fungal cells to kill off the fungal organism without dangerous effects on the host. Unlike bacteria, both fungi and human cells are eukaryotes. Thus, fungal and human cells are similar at the molecular level, making it more difficult to find a target for an antifungal drug to attack

that does not also exist in the infected organism. Consequently, there are often side effects to some of the antifungal drugs. Some of these side effects can be life-threatening if the drug is not used properly. Antifungal agents include:

2.2.2.1 Azoles

Azole based antimycotic agents include Fluconazole and Ketoconazole. They act by inhibiting 14ά-demethylation of lanosterol in the ergosterol biosynthetic pathway. The primary target for azoles is the heme protein, which co- catalyzes cytochrome P- 450-dependant 14ά-demethylation of lanosterol. Inhibition of 14ά-demethylase leads to depletion of ergosterol and accumulation of sterol precursors, resulting in the formation of a plasma membrane with altered structure and function (Ghannoum and Rice, 1999).

2.2.2.2 Polyenes

This includes Ampotericin B. These act by binding to ergosterol on the fungal membranes, thereby perturbing membrane functions. This leads to altered permeability, leakage of vital cytoplasmic components and death of the organism (Odds *et al*., 2003).

2.2.2.3 Flucytosine (5- Fluorocytosine)

Flucytosine is a fluorinated pyrimidine with inhibitory activity against many types of yeast. It works as an antifungal agent through conversion to 5- fluorouracil, which gets incorporated into fungal RNA. This causes premature chain termination thereby inhibiting DNA synthesis through effects on the thymidylate synthase. Thus flucytosine acts by interfering with pyrimidine metabolism, as well as RNA, DNA, and protein synthesis in the fungal cell (Odds *et al*., 2003; Ghannoum and Rice, 1999).

2.2.2.4 Echinocandins

These are fungal secondary metabolites comprising of a cyclic hexapeptide core with a lipid side chain responsible for antifungal activity. They inhibit synthesis of 1,3–β-D-glucan, a fundamental component of the fungal cell wall. Inhibition is effective and brief exposure leads to cell death (Odds *et al*., 2003).

2.3 ANTIMICROBIAL DRUG RESISTANCE

Microbiological resistance refers to non-susceptibility of a fungus/ bacteria to an antifungal agent/antibiotic by *in vitro* susceptibility testing, in which the MIC of the drug exceeds the susceptibility breakpoint for that organism (Kanafani and Perfect, 2008). Antimicrobial resistance is a major public health threat associated with increased

morbidity and mortality as well as enormous healthcare costs attributed to longer hospital stays (Mainous and Pomeroy, 2010).

Antimicrobial drug failure may occur due to many reasons such as reduced adherence to drug therapy, suboptimal dosing, diagnostic and laboratory error, ineffective infection control, counterfeit or altered drugs, and resistance (innate or acquired) (Kunin, 1995).

Resistance to antimicrobials occurs in four main ways. The first possible mechanism is the mutation of the drug's target. A second mechanism is a bypass of the drug's target by the acquisition of a similar but insensitive target protein. A third mechanism is the enzymatic degradation or modification of the drug. And fourth, resistance can be caused by a nonspecific reduced permeability to antimicrobial drugs. This is typically caused by reduced production of porins, the protein channels that allow antimicrobials through the outer bacterial membrane, and/or an increased production of drug-efflux pumps, which remove drugs from both cytoplasm and periplasm (Avison, 2005; Poole, 2005).

Antimicrobial drug resistance is commonly described in terms of the resistance characteristics of the microorganism. These characteristics are either intrinsic to the

organism (Primary) or resistance factors acquired (Secondary) through induced genetic expression or gene transfer between organisms (MacPherson *et al*., 2009)

2.3.1 Antibacterial drug resistance

Antibiotic resistance was recognized soon after the discovery of antibiotics. Diseases that were once thought to be controlled by antibiotics are returning in new leagues resistant to these therapies. Table 2.1 summarizes resistance mechanisms of the different groups of antibiotics.

Methicillin was developed and introduced in the 1960s to circumvent inactivation by the common beta-lactamases that caused penicillin resistance. But methicillin- resistant *Staphylococcus aureus* (MRSA) emerged soon after to combat the beta-lactamase resistant penicillin analog. Resistance to Vancomycin, the antibiotic of choice for treating multidrug-resistant MRSA, has also been reported (Hiramatsu *et al*., 1997).

Multiple resistance organisms render therapy more costly and sometimes unsuccessful. Individuals may succumb to multidrug resistant (MDR) infections because all available drugs have failed, especially in the developing world. Notable global examples include hospital and community MDR strains of *Mycobacterium tuberculosis*, *Enterococcus*

faecium, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Acinetobacter baumanii* and *Pseudomonas aeruginosa*. In developing countries, MDR enteric disease agents such as *Salmonella enteritidis*, *Shigella flexneri* and *Vibrio cholerae* are a threat to the public (Levy and Marshall, 2004).

Table 2. 1: Resistance mechanisms of antibiotics

Antibiotic(s)	Mechanisms	Genetic basis
β- lactams	Altered penicillin-binding proteins	Chromosomal
	Reduced permeability	Chromosomal
	β- lactamase	Chromosomal and plasmid
Fluoroquinolones	Altered DNA gyrase	Chromosomal
	Reduced permeability	Chromosomal
Aminoglycosides	Decreased ribosomal binding	Chromosomal
	Reduced uptake	Chromosomal
	Modifying enzymes	Plasmid
Macrolides	Methylating enzymes	Chromosomal and plasmid
Tetracyclines	Efflux	Plasmid
	Ribosomal protein altered	Plasmid

Table courtesy Neu, 1992.

2.3.2 Antifungal drug resistance

Antifungal drug resistance is a prominent feature in the management of invasive mycoses. Non-susceptibility of fungus to antifungal agents has been observed in virtually all classes of antifungal agents.

Azole resistance has been reported in *Candida* spp and *Aspergilus* spp with *Candida krusei* being intrinsically resistant to Fluconazole (Goldman *et al*., 2000). Widespread use of itraconazole and fluconazole is thought to have been the major driver of azole resistance.

Echinocandins are highly effective against *Candida* and *Aspergillus* species, but they have no activity against zygomycetes or against *Cryptococcus*, *Trichosporon*, *Scedosporium*, and *Fusarium* species (Espinel-Ingroff, 2003).

Resistance to Amphotericin B among *Candida* strains is rare. However, intrinsic polyene resistance is frequently noted in *Candida lusitaniae* and *Trichosporon beigelii* (Kanafani and Perfect, 2008).

2.4 THERAPEUTIC POTENTIAL OF PLANTS AS SOURCE OF NEW ANTIMICROBIALS

Plants and their products have been used for centuries to prevent and treat many diseases. According to the world health organization (WHO, 2002), about 80% of the population in Africa relies on traditional remedies (mainly herbs) for the healthcare of its people mainly due to its accessibility and affordability. Currently, there is an urgent need to identify novel, active compounds as leads for effective drug development. This is because of the widespread resistance of microorganisms to most drugs with adverse side effects reported with use of some drugs such as Amphotericin B (Sterling and Merz, 1998).Plant derived compounds of medicinal value have made large contributions to human health. Their role in the development of new drugs is through either becoming the base for the development of a medicine, a natural blueprint for the development of new drugs, or; a phytomedicine to be used for the treatment of disease (Jachak and Saklani, 2007).

It has been estimated that only 5-15% of the approximately 250 000 species of higher plants have been systematically investigated for the presence of bioactive compounds (Balandrin *et al*., 1993). An estimated 122 drugs from 94 plant species have been discovered through ethnobotanical studies (Fabricant and Farnsworth, 2001). These include Berberine, Emetine and Quinine among others as shown in Table 2.2. This is an

indication that there is still an abundance of drugs yet to be discovered from plants. More than a quarter of all the medicines used in the world today contain ingredients derived from plants (Ma *et al*., 2005).

Table 2. 2: Some Common Plants Derived from Plants

.Drug	Action or clinical use	Plant source
Acetyldigoxin	Cardiotonic	*Digitalis lanata* Ehrh.
Aesculetin	Antidysentery	*Fraxinus rhynchophylla* Hance
Berberine	Bacillary dysentery	*Berberis vulgaris* L.
Colchicine	Antitumor agent; antigout	*Colchicum autumnale* L.
Deserpidine	Antihypertensive; tranqulizer	*Rauvolfia canescens* L.
Emetine	Amoebicide; emetic	*Cephaelis ipecacuanha* (Brotero) A. Richard
Quinine	Antimalarial	*Cinchona ledgeriana* Moens ex. Trimen
Theophylline	Diuretic; bronchodilator	*Camellia sinensis* (L.) Kuntze
Morphine	Analgesic	*Papaver somniferum* L.

Data adapted from Fabricant and Farnsworth, 2001.

2.5 ANTIMICROBIAL COMPOUNDS FROM PLANTS

Plants are rich in a wide variety of secondary metabolites which serve as defense mechanisms against predation by microorganism, insects and herbivores. Useful major groups of antimicrobial phytochemicals can be divided into several categories.

2.5.1 Alkaloids

These are heterocyclic nitrogenous compounds, known to have microbiocidal effects. Some key examples are Berberine, Morphine and Codeine. Solamargine, a glycoalkaloid from berries of *Solanum khasianum* have been found to have antidiarrheal effects (Cowan, 1999). Diterpenoid alkaloids isolated from plants of the Ranunculaceae family have been found to have antimicrobial properties (Ramar and Ponnalpanam, 2008).

2.5.2 Flavones

These are phenolic compounds containing one carbonyl group. They are known to be synthesized by plants in response to microbial infections and are effective substances against a wide range of microorganisms (Ramar and Ponnalpanam, 2008). Their activity is due to their ability to complex with extracellular and soluble proteins and also

bacterial cell walls. Examples include Catechin found in green tea and Phloretin found in some apples (Cowan, 1999).

2.5.3 Essential oils and terpenoids

Plant essential oils possess broad spectrum antimicrobial activities attributed to the high content of phenolic derivatives such as thymol and carvacrol. Some essential oils are used for systemic and superficial fungal infections. Fragrance of plants is associated with essential oils. The oils consist of secondary metabolites which are enriched with terpenes. Terpenes are active against both bacteria and fungi (Ahamd *et al*., 1993).

2.5.4 Phenolics

These are compounds with a phenolic ring such as Cinnamic and Caffeic acids. Phenols are toxic to microorganisms because of the sites and numbers of hydroxyl groups on the phenol groups, which is also related to their relative toxicity to microorganisms. Their toxicity is through enzyme inhibition by the oxidized compounds through reaction with sulfhydryl groups or through more non-specific interactions with proteins (Ramar and Ponnalpanam, 2008).

2.5.5 Quinones

They are compounds having aromatic rings with two ketone substitutions and are ubiquitous in nature. These compounds are responsible for the browning reactions in cut or injured fruits and vegetables. Quinones have been shown to have antimicrobial activity with hypericin and an anthraquinone from *cassia italic* as examples (Cowan, 1999). Quinones are known to complex irreversibly with nucleophilic amino acids in proteins leading to inactivation of proteins and loss of functions (Stern *et al*., 1996).

2.6 REVIEW OF PLANTS STUDIED

In this study, four plants were considered. The selection was based on the fact that they are used traditionally to treat symptoms related microbial infections.

2.6.1 *Hagenia abyssinica* (Bruce) J.F.Gmel

Hagenia abyssinicca is a tree that grows to 5-25m high (Figure 2.2). It belongs to the family Rosaceae and is known in vernacular as; *Bondet* (Kipsigis), *Mujogajoga* (Meru), *Omukunakuna* (Kisii), *Muinyeri* (Kikuyu) (Kokwaro, 1976).

Figure 2. 2: *Hagenia abyssinica* tree.

Traditionally, the bark is used to treat diarrhoea, stomach- ache and as an anthelminthic, while the root is used for treatment of malaria and general illnesses (Kokwaro, 1993). Female flowers of the plant are used for removing intestinal worms (Beentje, 1994**).** The essential oil of the female flower of *H. abyssinicca* has been found to have trypanocidal and cytotoxic activity with Ledol, a compound isolated from the flower being identified as the main component of the essential oil (Nibrent and Wink, 2010). Phytochemical studies of both male and female flowers have shown the presence of phloroglucinols and phenolic acids (Woldemariam *et al*., 1990). The kosins (phloroglucinol derivatives) exhibited cytotoxic activity in vitro and in vivo against a

panel of three transplantable murine adenocarcinomas of the colon (Woldemariam *et al*., 1992).

2.6.2 *Fuerstia africana* T.C.E. Fries

The herb (Figure 2.3) belongs to the family Labiatae and is known in vernacular as; *Birirwob-Sot* (Kipsigis), *Kalaku* (Kamba), *Kimamuo* (Chagga), Oloito-dor aik (Maasai) (Kokwaro, 1976).

Figure 2. 3: *Fuerstia africana* (Ticah interactive guide. com).

Traditionally, the leaves are reported to treat stomach ulcers, tongue infection, conjuctivities, malaria, gonorrhoea, skin ailments, colds and in prevention of diarrhea (Kokwaro, 1993; Githinji and Kokwaro, 1993; Koch *et al* 2005; Muganga *et al*., 2010). The plant has been reported to have antiplasmodial activity both *in vitro* and *in vivo* (Koch *et al*., 2005; Muthaura *et al*., 2007; Muganga *et al*., 2010).

2.6.3 *Ekebergia capensis* Sparrm.

This is a tree that grows up to 30m high (Figure 2.4). It belongs to the family Meliaceae and is known in vernacular as; *Araruet* (Kipsigis), *Mukongui* (Kamba), *Omonyamavi* (Kisii), *Tido* (Luo), *Muchogomo* (Meru) (Kokwaro, 1976).

Figure 2. 4: Photo of *E. capensis* Tree and fruits (Fernkloof.com).

E. capensis Bark has been reported to be used traditionally for treating sores, abscesses boils, heartburn, dysentery, acne and malaria (Rabe and Van Staden, 1997; Koch *et al* 2005; Kamadyaapa *et al*, 2009). The roots are used for treating scabies,colds and headache, (Verschaeve and Van Staden, 2008)

The plant has been reported to be active against drug resistant strains of *M. tuberculosis* and have antimalarial activity (Lall and Meyer, 1999; Murata *et al*., 2008).

2.6.4 *Asparagus racemosus* (Willd.)

Asparagus racemosus is a herb employed in traditional medicine in many parts of the world. Figure 2.5 illustrates the herb. It belongs to the family Asparagaceae/Liliaceae. Its vernacular names include; *Ketabelelit* (Kipsigis), *Karura* (Embu) (Kokwaro, 1976). The roots of *A. racemosus* are traditionally used to treat indigestions and gonorrhoea, bilharzia (Kokwaro, 1976; Gachathi, 1989).

Its medicinal usage has been reported in the Indian and British Pharmacopoeias and in indigenous systems of medicine. It's recommended in Indian Ayurvedic texts for prevention and treatment of gastric ulcers, dyspepsia and as a galactogogue. It is also used successfully for nervous disorders, inflammation, liver diseases among other infectious diseases. The juice of fresh roots has curative effect in patients with duodenal ulcers. Oral administration of decoction of powered root enhances the immuno-modulatory effect (Bopana and Saxena, 2007).

Figure 2. 5: Photo of *A. racemosus* herb and harvested roots.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 STUDY SITE

The study was carried out at the Center for Traditional Medicine and Drug Research (CTMDR) and Center for Microbiology Research (CMR) KEMRI, Nairobi.

3.2 EXPERIMENTAL DESIGN

The study was laboratory based. Four plants were selected for the study and they were *Hagenia abyssinica* (Bruce) J.F.Gmel (Leaves and Stem barks), *Fuerstia africana* T.C.E. Fries (Aerial parts), *Asparagus racemosus* (Willd.)(Roots) and *Ekebergia capensis* Sparrm. (Stem bark).

Four extracts were obtained for each plant sample. The extracts obtained were hexane, dichloromethane, methanol and water, giving a total of twenty extracts from the five plant samples.

Eleven microorganisms were used for the antimicrobial assays, five bacteria and six fungi. Extracts were tested at a concentration of 1000µg/disc. Antimicrobial assays

were carried out for each extract in triplicate and results compared against the standard drug. Chloramphenical was used as the standard for bacterial assays while Miconazole and Fluconazole for fungal assays. Dissolution solvent (DMSO) and extraction solvents (Hexane, Dichloromethane, Methanol) were used as negative controls.

Toxicity studies were carried out on active extracts. Cytotoxicity studies using VERO E6 cancer cell lines were used to determine toxicity of extracts *in vitro*. Extracts with IC_{50} > 90µg/ml was classified as not cytotoxic. Acute toxicity was determined using Swiss Albino mice at two dose levels, 2500and 5000mg/kg/body weight.

3.3 MATERIALS

3.3.1 Plants

Four plant species were selected based on available ethnobotanical information from literature as shown in Table 3.1. The plants were *Hagenia abyssinica* (Bruce) J.F.Gmel, *Fuerstia africana* T.C.E. Fries, *Asparagus racemosus* (Willd.), and *Ekebergia capensis* Sparrm.

The plants were collected from their natural habitats in two regions within Rift valley province, *H. abyssinica, A. racemosus* and *E. capensis* from Olenguruone in Molo district, while *F. africana* from Cheptenye in Kericho district.

Authentication of the plant species was done by Mr. Patrick Mutiso from University of Nairobi and samples were assigned voucher specimen numbers 2009/001-004, then deposited at the University of Nairobi Herbarium. Information on plants and parts collected are shown in Table 3.2.

Table 3. 1: Plants selected for the study

Botanical name of plant	Family	Vernacular names	Parts used	Ethno botanical uses
***Hagenia abyssinica* (Bruce) J.F. Gmel**	Rosacea	Bondet (Kipsigis), Mujogajoga (Meru), Muinyeri (Kikuyu), Omukunakuna (Kisii).	Bark, Roots, Female flowers	Treats diarrhoea, stomach ache, malaria, & as an antihelminthic (Kokwaro, 1976, Beentje, 1994)
***Fuerstia africana* T.C.E. Fries**	Labiatae	Birirwob- Sot (Kipsigis), Kalaku (Kamba), Oloito-dor-aik (Maasai).	Leaves	Treats stomach ulcers, tongue infection, malaria, diiarhoea & as an antihelminthic (Kokwaro, 1976, Beentje, 1994)
***Ekebergia capensis* Sparrm**	Meliaceae	Araruet (Kipsigis), Tido (Luo), Muchgomo (Meru)	Bark, Roots	Treats sores, malaria dysentry, scabies, acne, headache (Kokwaro, 1976, Beentje, 1994)
***Asparagus racemosus* (Willd.)**	Asparagaceae/ Liliaceae	Ketabelelit (Kipsigis), Karura (Embu)	Roots	Treats indigestion, gonorrhoea, bilharzia (Kokwaro, 1976, Beentje, 1994).

Table 3. 2: Plants collected, parts collected and voucher specimen numbers

Plant species	Parts collected	Voucher Specimen Numbers
Hagenia abyssinica	Leaves, Stem bark	2009/004
Fuerstia africana	Aerial Parts	2009/003
Asparagus racemosus	Roots	2009/001
Ekebergia capensis	Stem Bark	2009/002

3.3.2 Extraction solvents

Solvents used for extraction were of analytical grade. These include hexane (Merck), dichloromethane (Merck) and methanol (Merck).

3.3.3 Microorganisms used

Microorganisms used were both standard reference strains and clinical isolates preserved at -20ºC. The isolates were obtained from Center for Microbiology Research, KEMRI (Kenyatta Hospital), courtesy of Dr. Christine Bii.

Bacterial strains selected for the study included Gram positive *Staphylococcus aureus (*ATCC 25923) and Methicillin resistant *Staphylococcus aureus* (clinical isolate) while Gram negative bacteria included *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (clinical isolate) and *Pseudomonas aeruginosa* (ATCC 27853).

Fungal strains selected for the study included both yeasts and dermatophytes. The yeasts were *Candida albicans* (ATCC 90028), *Candida krusei* (ATCC 6258), *Candida parapsilosis* (ATCC 22019) and *Cryptococcus neoformans* (ATCC 66031), while dermatophytes were *Trichophyton mentagrophytes* (clinical isolate) and *Microsporum gypseum (*clinical isolate).

3.3.4 Microbial culture media and preparation

Both Sabouraud dextrose and Mueller Hinton agar were used. Sabouraud Dextrose Agar (Oxoid) (SDA) was used as growth media for the fungal strains while Mueller Hinton agar (Oxoid) (MH) was used for the bacterial strains. Media for the experiment was prepared as per the manufacturer's guidelines indicated on the labels. SDA was prepared by dissolving 68 g of SDA powder in 1L of distilled water, then autoclaved at 121ºC for 15 minutes. The agar was then poured onto sterilized and labeled culture plates while

still hot and left to cool and solidify in a clean bench, then stored at 4º C. A similar procedure was used to prepare MH agar with 38 g of MH agar powder used.

3.3.5 VERO E6 Cell lines

VERO E6 cancer cell lines sourced from Toyoma Medical and Pharmaceutical University, Toyoma Japan, were obtained from Biological sciences unit at the CTMDR, KEMRI. The cell lines originate from the African green monkey and were used for *in vitro* cytotoxicity assays. The cells were cultured and maintained in Eagle's minimum essential media (MEM) (Gibco, BRL, Scotland), supplemented with 10% fetal bovine serum (FBS) (Gibco, BRL, Scotland) in 25ml cell culture flasks incubated at 37º C in 5% CO_2.

3.3.6 Rodents used

Swiss female mice aged 6-8 weeks old, weighing 20 ± 2 g were obtained from the animal house facility at KEMRI. The mice were bred in standard macrolon type II cages and fed with the standard diet and water *ad libitum*.

3.4 METHODS

3.4.1 Preparation of plants and extraction of crude extracts

The plant materials collected were air dried by spreading in the open under shade at room temperature (25ºC) for two weeks. The plant samples were ground separately into fine powder using an electric laboratory mill. Powders were packed in plastic bags, weighed and stored at room temperature until further use.

Both non-polar and polar extracts were prepared for each sample. Non-polar extracts were prepared by extracting 100 g of plant sample with the exception of *A racemosus* 50 g and *F africana* 45 g whose total plant material weight decreased after plant drying. Extraction was carried out successively with 200ml of hexane, dichloromethane and methanol respectively (Muthaura *et al.*, 2007). The extracts were filtered through Whatman No. 1 filter paper and the filtrate after each successive extraction was concentrated under reduced pressure at 40ºC using a BÜCHI, Switzerland, rotary evaporator. The resultant extract was weighed and stored in airtight sample bottles at room temperature.

Water extracts were prepared by soaking in a conical flask 100g of each plant powder in 200ml of distilled water with the exception of *A. racemosus* 28g, *H. abyssinica* stem

bark 65g and *F. africana* 34g. The flasks were placed in a water bath set at 60ºC for 2 hours. The extract was filtered through cotton gauze into a round bottomed flask. The filtrate of each extract was then frozen by placing in the round bottomed flask in a bowl containing a mixture of acetone and dry ice, then attached to a freeze dryer (Modulyo, Edwards). The resultant aqueous extract was weighed and stored until required for bioassays (Harborne, 1984).

The percentage yields for extracts were determined according to Kigondu *et a*l., 2009 as

$$\frac{\text{Weight of extract}}{\text{Weight of plant material}} \times 100 = \text{Percentage yield.}$$

Exactly 100 mg of each extract was weighed and put into a sterilized sample bottle and then dissolved in 1ml of 100 % DMSO (Sigma) for non-polar extracts while for aqueous extracts, sterilized distilled water, to make a concentration of 100 mg/ml and stored at 4ºC.

3.4.2 Antimicrobial assay

To activate the strains, a loop-full of each microbial strain was inoculated onto appropriate media and incubated at 37º C for 24 h for bacteria and 35º C and 30ºC for 72 h for yeast and molds respectively.

The agar diffusion method (Singh *et al.,* 2006) was used to evaluate the antimicrobial activity. Bacteria were cultured overnight at 37º C in Mueller Hinton Agar (MHA) and fungi at 28º C for 72 h in Sabouraud Dextrose Agar (SDA) and used as inoculums. Test plates were prepared with MHA and SDA and inoculated on the surface with a cell suspension in sterile normal saline. In all cases, the concentration of the inoculum was adjusted to 1.5×10^{8} CFU/mL.

The standard drugs used were Chloramphenical (Beckton Dickinson) (30 µg/ disc) for bacteria, Miconazole (MP Biomedicals) (27.3 µg/ disc) and Fluconazole (25 µg/disc) for fungi. Negative controls were the solvents used in extraction (hexane, dichloromethane and methanol), and dissolution (DMSO).

Paper discs prepared from Whatman No 1 and sterilized in an autoclave were used for the disc diffusion assay. The disc (6mm in diameter) was impregnated using a pipette with 10 µl of extracts (1000 µg/disc) from a stock solution of 100 mg/ml and placed on agar. Discs of Chloramphenicol (30 µg) was used as standard for bacteria while Miconazole (27.3 µg/disc) and Fluconazole (25 µg/disc) for fungi. Discs containing solvents used in extraction (hexane, dichloromethane and methanol), and dissolution (DMSO) were used as negative controls. The test plates were incubated at 37º C for 24 h for bacteria and at 35º C and 30º C for 72 h for both yeasts and dermatophytes respectively. Triplicate assays were carried out for each extract. Inhibition zones were measured in millimeters and results expressed as mean inhibition zones of the three assays. Antimicrobial activity was classified as ranging from little or no activity at ≤ 10 mm to very strong activity for inhibition zone diameters of ≥ 30 mm (Table 5) according to Lee *et al*., 2004.

Table 3. 3: Classification of degree of antimicrobial activities

Activity	Inhibition zone diameters in millimeters
Very strong	≥ 30
Strong	21 – 29
Moderate	16 – 20
Weak	11 – 15
Little or No activity	≤ 10

Lee *et al*., 2004.

The Minimum Inhibitory Concentrations (MIC) was determined for extracts which had inhibition zones of ≥8 mm against the test microorganisms. Sterile filter paper discs (6 mm in diameter) containing1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91 and 1.95 µg/disc of dissolved plant extracts were placed on the surface of appropriate medium inoculated with test microorganism. MIC was defined as the lowest concentration of extract that inhibited visible growth of microorganism on agar.

3.4.3 Cytotoxicity assay

The extracts of the most active plants were tested for *in vitro* cytotoxicity, using VERO E6 cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). Cells were maintained in Eagle's minimum essential medium (MEM) supplemented with 10 % fetal bovine serum (FBS). The cells were cultured in 25ml cell culture flasks at 37º C in 5% CO_2. Upon attaining confluence, the cells were detached by trypsinization. 100µl cell suspension (1×10^5 cells/ml) was added to each well in a 96-well micro-titer plate. The extracts were assayed in duplicate and the cells incubated at 37º C in 5% CO_2 for 24 h in order to attach. The medium was aspirated off from row H, 150µl of the highest concentration of each of the extract (a serial dilution, prepared in MEM) added into the same row and a threefold serial dilution done up to row B yielding a concentration range from 100 µg/ml to 0.14 µg/ml. The experimental plates were incubated further at 37 ºC for 48 h. The cells in media without drugs were used as controls. MTT (10µl) was added into each well and the cells incubated for 4 h, until a purple precipitate was clearly visible under a microscope, the medium together with MTT was aspirated off from the wells, DMSO(100µl) added and the plates shaken for 5 min. The plates were then read (colour absorbence) on an ELISA scanning multiwell spectrophotometer (Multiskan Ex labssystems) at 562 nm and 690 nm as reference.

Data was analysed as follows:

$$\% \text{ Cell viability} = \frac{[OD_{sample562} - OD_{690}]}{OD_{control562} - OD_{690}} \times 100$$

Where OD = optical density

Data was transferred onto a graphic programme (EXCEL) and expressed as percentage of the untreated controls. The 50% inhibition concentration (IC_{50}) value was evaluated by linear regression analysis. Chloroquine (CQ) was used as the reference drug for the experiment. Extracts were classified as cytotoxic if their $IC_{50} < 90$ µg/ml (Irungu *et al*., 2007)

3.4.4 Determination of acute toxicity

Authorization to use mice for *in vivo* acute toxicity was granted by the KEMRI Animal Care and Use Committee (ACUC). Healthy Swiss female mice, 8 weeks old weighing 20 ±2 g were randomly divided into groups of five in each cage. The extracts were dissolved in a solution of 10% Tween 80 in double distilled water and administered by gavage at a dose of 2500 and 5000 mg/kg body weight of mice/0.2ml. The mice had access to tap water and food, except for a short fasting period (12 h) before oral administration of 0.2 ml of the extract to each mouse (Muthaura *et al.,* 2007). The

general behavior of mice was observed continuously for 1 h after the treatment and then intermittently for 4 h, and thereafter over a period of 24 h. The mice were further observed for up to 14 days following treatment for any signs of toxicity such as food intake, activity, tremors, and the latency of death. Weights of surviving mice were taken on day 14.

3.4.5 Phytochemical screening of most bioactive plant extracts

Plant extracts exhibiting biological activity were screened for presence of phytochemical constituents using thin layer chromatography (TLC). The chromatographic tank was allowed to equilibrate with the solvent system (Chloroform: Methanol - 98:2, with 5 drops of glacial acetic acid), for at least 30 minutes prior to development of plates. The plates were then sprayed with TLC visualization agents that give specific reactions (Harbone, 1984). Presence of different classes of compounds was determined by observing appropriate color change on the TLC plate (Table 6).

Table 3. 4: TLC Visualization reagents for different groups of compounds and expected observation

Group of Compounds	Visualization reagent	Expected observation
Terpenoids	Vanillin sulphuric acid	Purple color spots
Alkaloids	Dragendorff reagent	Orange-brown spots on a yellow background
Flavones	Ammonia fumes	Yellow-brown spots
Phenolics	Ferric- Ferricyanide	Blue spots
Anthraquinones	Methanolic potassium hydroxide (Kedde reagent) and observation under Ultraviolet light.	Yellow-brown spots change to red, violet, green or purple under Ultraviolet light (254nm).

Harbone, 1984.

3.4.6 Data analysis

The *in vitro* antimicrobial assays, inhibition zone diameters were determined for 3 replicate experiments and results expressed as mean inhibition zones. The results were compared to the standard drug mean inhibition zones for each test pathogen using the student's *t*-test. A *p* – value of less than or equal to 0.05 was statistically defined as significant. The StatView computer software by SAS Institute Inc. USA was used for the statistical analysis. The 50% inhibitory concentration (IC_{50}) for the *in vitro* cytotoxicity assay was determined by plotting a dose response curve of percentage cell viability on EXCEL. The IC_{50} was determined by linear regression analysis.

CHAPTER FOUR

4.0 RESULTS

4.1 EXTRACTION OF PLANT CRUDE EXTRACTS

The powdered plant samples were extracted in hexane, dichloromethane (non- polar) methanol and water (polar) resulting in a total of twenty crude extracts. The trend in yield showed increase in percentage yield with increase in polarity of the extracting solvent (Table 4.1, Figure 4.1). The highest yields were encountered in water extracts while the lowest in hexane extracts. The roots of *Asparagus racemosus* had the highest yields in both water and methanol extracts with percentage yields of 27.96% and 9.57% respectively. The dichloromethane extract of leaves of *Hagenia abyssinica* had the highest yield of 1.35% among the dichloromethane extracts while hexane extract of *Fuerstia africana* had the highest yield in hexane extracts. The lowest percentage yields recorded was the hexane extract of the stem bark of *Hagenia abyssinica* and *Ekebergia capensis* with yields of 0.09% each.

Table 4. 1: Percentage yields of plant extracts using different solvents

Plants/parts	**Percentage yields(%) of extracts**			
	Hexane	**Dichloromethane**	**Methanol**	**Water**
Hagenia abyssinica (SB)	0.09	0.33	4.07	5.22
Hagenia abyssinica (L)	0.47	1.35	3.58	10.57
Ekerbagia capensis (SB)	0.09	1.02	3.38	5.32
Asparagus racemosus (R)	0.1	0.4	9.57	27.96
Fuerstia africana (A)	0.65	1.23	3.43	9.42

Stem bark (SB), Leaves (L), Roots (R), Aerial parts (A).

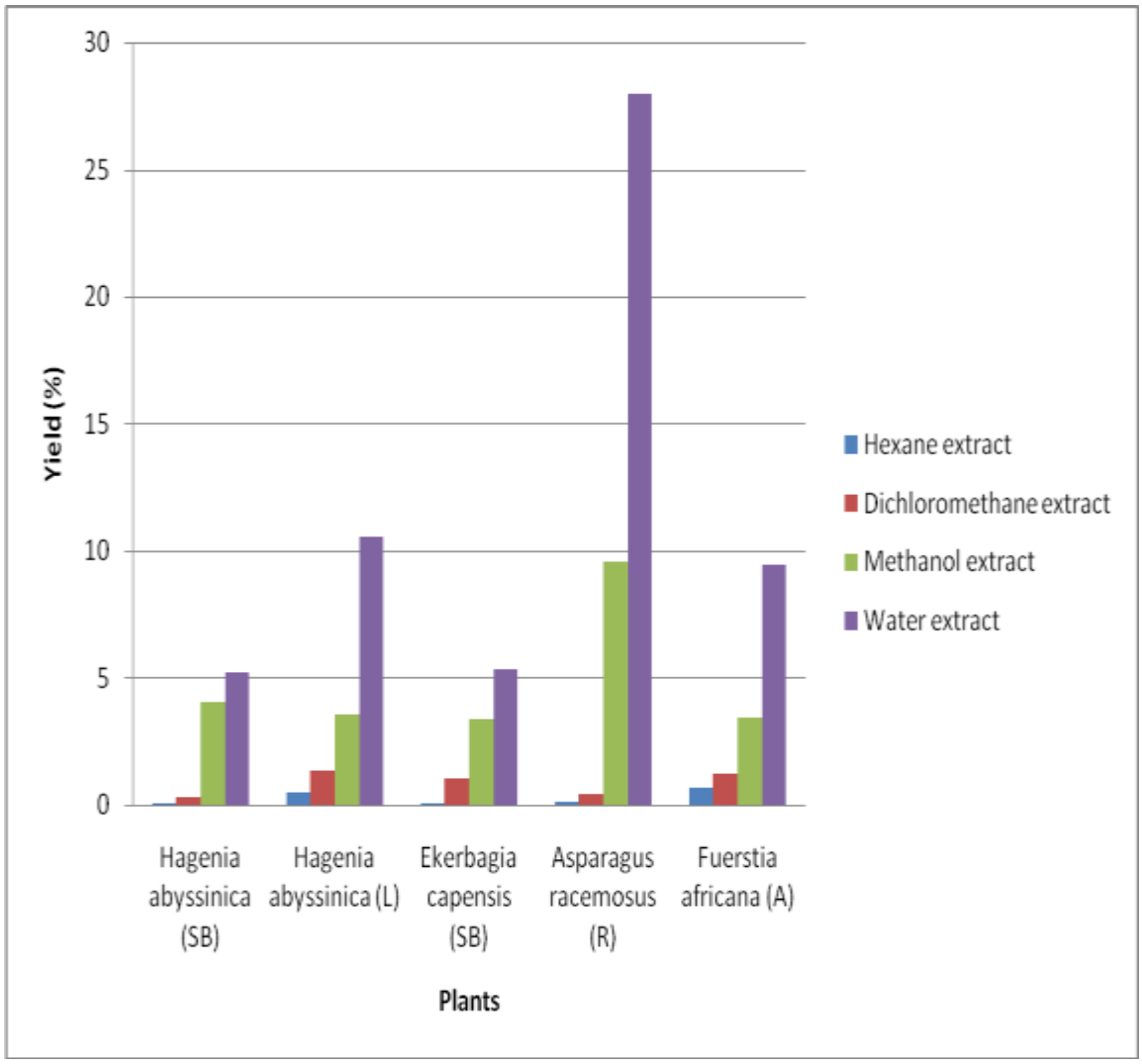


Figure 4. 2: Comparison of yields of different plant extracts with solvents used**.**

A chart showing percentage yield of five plants samples from four plants after extracting with various solvents.

4.2 EVALUATION OF ANTIMICROBIAL ACTIVITY OF PLANT CRUDE EXTRACTS

4.2.1 Antibacterial activity

Antibacterial activity results were expressed as means of three replicates (Appendix 1). The crude extracts of the four plants selected had inhibitory effects on several test organisms (Table 4.2) *P. aeruginosa* was inhibited by 65% of extracts tested, followed by *S. aureus* (55%), then MRSA (50%) and lastly *K. pneumoniae* (15%). *E. coli* was resistant to all the extracts tested.

The dichloromethane extract of leaves of *H. abyssinica* was the most active extract with the highest zone of inhibition of 20 mm against *S. aureus* and 19.5 mm against MRSA indicating moderate activity. This was higher in comparison to the reference drug Chloramphenical, which had inhibition zones of 17.33 mm and 15.0 mm against *S. aureus*, and MRSA respectively. The extract also inhibited *P. aeruginosa* with zone diameter almost equal to the standard drug. However, this difference was not statistically significant ($p > 0.05$). Hexane extract of the leaves of *H. abyssinica* was also moderately active against *S. aureus* and MRSA. The hexane extract had an inhibition zone diameter of 19.33 mm against MRSA which was higher than that of the reference drug Chloramphenical of 15 mm. The extract had an inhibition zone of 16.67 mm against *S. aureus.*

Water extracts of all plants were the least active with the highest activity observed in the stem bark of *H. abyssinica*, having an inhibition zone diameter of 11.33 mm against *P.* a*eruginosa*, followed by *E. capensis* with inhibition zone diameter of 10.5 mm against MRSA. Both were classified as having weak activity, with the rest of the extracts having little or no activity.

Gram positive bacteria were more susceptible to the extracts than gram negative bacteria. Three gram negative bacterial strains were used in the study. The plant extracts inhibited *P. aeruginosa* with inhibition zones of up to 15.5 mm. No inhibition zones were observed for extracts on *E. coli*, while for *K. pneumoniae*, inhibition zones observed were ≤ 8 mm.

Descriptive statistics for plant extracts against the bacterial strains are found in appendix 3 to 6.

Table 4. 2: *In vitro* antibacterial activity of the test plant extracts against selected bacteria

Solvent	Plant(part)	Conc.µg/disc	Mean(n = 3) Inhibition zones (mm)				
			Sa	**Mrsa**	**Pa**	**Kp**	**Ec**
	Chloramphenical	**30**	**17.33**	**15.00**	**16.5**	**29.67**	**18**
Hexane	*H. abyssinica* (SB)	1000	9.00[a]	9.50[a]	-	-	-
	H.abyssinica (L)	1000	16.67[b]	19.33[a]	13.00[a]	-	-
	F. africana (A)	1000	9.33[a]	9.33[a]	8.5[a]	-	-
	E. capensis (SB)	1000	-	-	-	-	-
	A. racemosus (R)	1000	-	-	-	-	-
DCM	*H. abyssinica* (SB)	1000	8.67[a]	9.33[a]	-	-	-
	H.abyssinica (L)	1000	20.00[b]	19.50[a]	15.5[b]	-	-
	F. africana (A)	1000	10.67[a]	10.50[a]	9.67[a]	-	-
	E. capensis (SB)	1000	7.50[a]	-	-	-	-
	A. racemosus (R)	1000	-	-	11.67[a]	6.83[a]	-

Inhibition diameters inclusive of the disc diameter of 6 mm. Selected bacteria: **Sa**, *Staphylococcus aureus* ; **Mrsa** Methicillin resistant *Staphylococcus aureus*; **Pa**, *Pseudomonas aeruginosa* ; **Kp**, *Klebsiella pneumoniae* ; **Ec**, *Escherichia coli* . – Represents no inhibition zones observed. No inhibition zones were observed on the negative controls used. Reference drug used was Chloramphenical. [a] $p<0.05$, [b] $p>0.05$ versus reference drug by Student's t-test.

Table 4. 2: *In vitro* antibacterial activity of the test plant extracts against selected bacteria

Solvent	Plant(part)	Conc.µg/disc	Mean(n = 3) Inhibition zones (mm)				
			Sa	**Mrsa**	**Pa**	**Kp**	**Ec**
	Chloramphenical	**30**	**17.33**	**15.00**	**16.5**	**29.67**	**18**
Methanol	*H. abyssinica* (SB)	1000	11.00[a]	10.75[a]	12.25[a]	-	-
	H.abyssinica (L)	1000	7.75[a]	7.63[a]	12.00[a]	-	-
	F. africana (A)	1000	6.75[a]	-	7.75[a]	-	-
	E. capensis (SB)	1000	11.00[a]	11.50[a]	10.67[a]	-	-
	A. racemosus (R)	1000	-	-	11.67[a]	-	-
Water	*H. abyssinica* (SB)	1000	-	-	11.33[a]	8.00[a]	-
	H.abyssinica (L)	1000	-	-	-	-	-
	F. africana (A)	1000	-	-	7.00[a]	-	-
	E. capensis (SB)	1000	-	10.50[a]	7.50[a]	-	-
	A. racemosus (R)	1000	-	-	-	7.00[a]	-

Inhibition diameters inclusive of the disc diameter of 6 mm. Selected bacteria for the test were: **Sa**, *Staphylococcus aureus* ; **Mrsa** Methicillin resistant *Staphylococcus aureus*; **Pa**, *Pseudomonas aeruginosa* ; **Kp**, *Klebsiella pneumoniae* ; **Ec**, *Escherichia coli* . – Represents no inhibition zones observed. No inhibition zones were observed on the negative controls used. Reference drug used was Chloramphenical. [a] $p<0.05$, [b]$p >0.05$ versus reference drug by Student's t-test.

4.2.2 Antifungal activity

All the extracts tested had either weak activity or little or no activity. Most extracts inhibited dermatophytes. The methanol extract of *A. racemosus* was the only extract that inhibited yeasts. Weak antifungal activity was observed in methanol extracts of *A. racemosus* and *E. capensis* with inhibition zone diameter of 12.33 mm against *T. mentagrophytes* for both plants. This was much lower than that of the reference drug Fluconazole which had an inhibition zone diameter of 24.33 mm. The methanol extract of *A. racemosus* had an inhibition zone diameter of 11.25 mm against *C. neoformans* and *M. gypseum*. The rest of the extracts tested were not active with inhibition diameters of < 10 mm. *C. albicans* and *C. parapsilosis* were resistant to all extracts tested. Antifungal activity of the test plants is summarized in Table 4.3.

No inhibition zones were observed for the negative controls. Descriptive statistics for plant extracts against the fungal strains are found in appendix 7 to 9.

Table 4. 3: *In vitro* antifungal activity of test extracts against selected fungi

Solvent	Plant(part)	Conc.µg /disc	Mean(n =3) Inhibition zones (mm)					
			Ca	**Ck**	**Cp**	**Cn**	**Tm**	**Mg**
	Miconazole	**27.3**	**17**	**10.67**	**17**	**15**	**Nd**	**Nd**
	Fluconazole	**25**	**Nd**	**Nd**	**Nd**	**Nd**	**24.33**	**25**
Hexane	*H. abyssinica* (SB)	1000	-	-	-	-	7.00[a]	-
	H. abyssinica (L)	1000	-	-	-	-	6.83[a]	-
	F. africana (A)	1000	-	-	-	-	7.83[a]	-
	E. capensis (SB)	1000	-	-	-	-	-	-
	A. racemosus (R)	1000	-	-	-	-	-	-
DCM	*H. abyssinica* (SB)	1000	-	-	-	-	-	-
	H.abyssinica (L)	1000	-	-	-	-	7.17[a]	-
	F. africana (A)	1000	-	-	-	-	7.17[a]	-
	E. capensis (SB)	1000	-	-	-	-	-	-
	A. racemosus (R)	1000	-	-	-	-	-	-

Inhibition diameters measured were inclusive of the disc diameter of 6mmSelected fungi were: **Ca**, *Candida albicans*; **Ck**, *Candida krusei*; **Cp**; *Candida parapsilosis*; **Cn**, *Cryptococcus neoformans*; **Tm**, *Trichophyton mentagrophytes*; **Mg**, *Microsporum gypseum* . **–** Represents no inhibition zones observed, **Nd** denotes not determined. No inhibition zones were observed on the negative controls used. [a] $p<0.05$ versus reference drug by Student's t-test

Table 4. 4: *In vitro* antifungal activity of test extracts against selected fungi

Solvent	Plant(part)	Conc.µg /disc	Mean(n =3) Inhibition zones (mm)					
			Ca	**Ck**	**Cp**	**Cn**	**Tm**	**Mg**
	Miconazole	**27.3**	**17**	**10.67**	**17**	**15**	**Nd**	**Nd**
	Fluconazole	**25**	**Nd**	**Nd**	**Nd**	**Nd**	**24.33**	**25**
Methanol	*H. abyssinica* (SB)	1000	-	-	-	-	-	7.33[a]
	H. abyssinica (L)	1000	-	-	-	-	-	-
	F. africana (A)	1000	-	-	-	-	7.33[a]	-
	E. capensis (SB)	1000	-	-	-	-	12.33[a]	8.00[a]
	A. racemosus (R)	1000	-	7.38[a]	-	11.25[a]	12.33[a]	10.67[a]
Water	*H. abyssinica* (SB)	1000	-	-	-	-	-	-
	H. abyssinica (L)	1000	-	-	-	-	-	-
	F. africana (A)	1000	-	-	-	-	-	-
	E. capensis (SB)	1000	-	-	-	-	-	-
	A. racemosus (R)	1000	-	-	-	-	-	-

Inhibition diameters inclusive of the disc diameter of 6mm. Selected fungi were: **Ca**, *Candida albicans*; **Ck**, *Candida krusei*; **Cp**; *Candida parapsilosis*; **Cn**, *Cryptococcus neoformans*; **Tm**, *Trichophyton mentagrophytes*; **Mg**, *Microsporum gypseum* . **–** Represents no inhibition zones observed, **Nd** denotes not determined. No inhibition zones were observed on the negative controls used. [a] $p<0.05$ versus reference drug by Student's t-test.

Figure 4. 3: Zones of inhibition of *C. neoformans.*

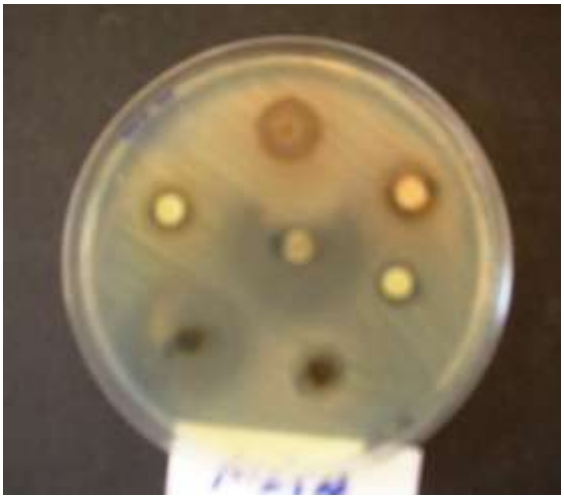


Figure 4. 4: Discs of extracts showing inhibition zones against MRSA.

4.2.3 Determination of Minimum Inhibitory Concentration (MIC)

The MIC of extracts was determined for extracts which had inhibition zones of ≥ 8 mm against the test microorganisms. A total of 13 extracts inhibited several microorganisms with inhibition zone diameters of ≥ 8 mm. The MIC was determined at concentrations ranging from 1000 – 1.95 µg/disc.

The lowest MIC values of < 1.95 µg/disc was exhibited the hexane extract of *H. abyssinica* leaves (MIC value of < 1.95 µg/disc) against *S. aureus* and MRSA. A similar observation was made for the methanol extract of *A. racemosus* which also showed an MIC value of < 1.95 µg/disc against *P. aeruginosa*. On the other hand, extracts of *E. capensis* had the least activity as their MIC values were high (250 to 1000 µg/disc) compared to the other plant species.

From the results, it is clear that the hexane and dichloromethane extracts of leaves of *H. abyssinica* leaves were the most active extracts as their MIC ranged from 31.25 to < 1.95µg/disc. This correlated to the high inhibition zone diameters which were equal or higher than those of Chloramphenical observed in the antibacterial assays. Results are summarized in Table 4.4.

Table 4. 5: Minimim Inhibitory Concentration of plant extracts with mean inhibition zone diameters of ≥ 8 mm

Plant species	Extract	Microorganisms tested MIC (µg/disc)					
		Sa	**Mrsa**	**Pa**	**Cn**	**Tm**	**Mg**
H. abyssinica (SB)	Hex	62.5	62.5	-	-	-	-
	Dcm	1000	1000	-	-	-	-
	Meth	250	500	62.5	-	-	-
	Water	-	-	62.5	-	-	-
H. abyssinica (L)	Hex	<1.95	<1.95	31.25	-	-	-
	Dcm	1.95	3.91	1.95	-	-	-
	Meth	-	-	125	-	-	-
F. africana (A)	Hex	62.5	62.5	31.25	-	500	-
	Dcm	31.25	31.25	500	-	-	-
E. capensis (SB)	Meth	250	250	250	-	500	1000
	Water	-	250	1000	-	-	1000
A. racemosus (R)	Dcm	-	-	500	-	-	-
	Meth	-	-	<1.95	1000	500	500
Chloramphenical		0.002	0.008	0.008			
Fluconazole					0.002	0.008	0.016

Microorganisms used denoted by **Sa**, *Staphylococcus* aureus ; **Mrsa** Methicillin resistant *Staphylococcus aureus*; **Pa**, *Pseudomonas aeruginosa* ; **Cn**, *Cryptococcus neoformans*; **Tm**, *Trichophyton mentagrophytes*; **Mg**, *Microsporum gypseum*; **-,** Not determined.

4.3 *IN VITRO* CYTOTOXICITY OF SELECTED PLANT EXTRACTS

Cytotoxicity against VERO cell line was determined for extracts having low MIC values of ≤ 500µg/disc (Table 4.5). Extracts were classified as cytotoxic if their IC_{50} < 90µg/ml.

Three out of eleven extracts were found to be highly cytotoxic with the fifty percent inhibitory concentration (IC_{50}) of < 8µg/ml. The extracts were hexane and dichloromethane extracts of leaves of *H. abyssinica* and dichloromethane extract of *F. africana*. This positively correlated with the extracts that had the lowest MIC values. The water extract of the stem bark of *H. abyssinica* was moderately cytotoxic with IC_{50} 31.56 µg/ml. Appendix 4 illustrates 96 well plates with extracts that were cytotoxic to VERO cell lines.

Table 4. 6: *In vitro* cytotoxicity of selected plant extracts on VERO cell line

Plant species (Plant part)	Extract	IC_{50} (µg/ml)
H. abyssinica (SB)	Hexane	>100
	Methanol	>100
	Water	31.56
H.abyssinica (L)	Hexane	7.84
	Dichloromethane	7.89
	Methanol	>100
F. africana (A)	Hexane	92.34
	Dichloromethane	7.91
E. capensis(SB)	Methanol	>100
	Water	>100
A. racemosus(R)	Methanol	>100
Chloroquine	Standard	43.39

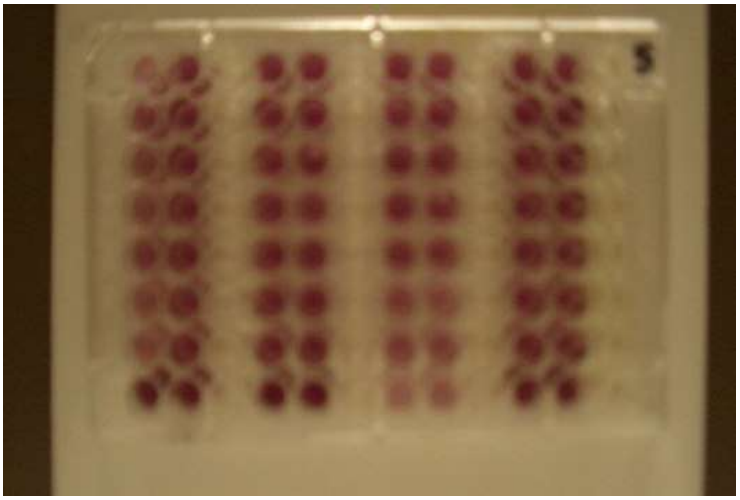


Figure 4. 5: Plate showing extracts that were not cytotoxic to Vero E6 cell lines.

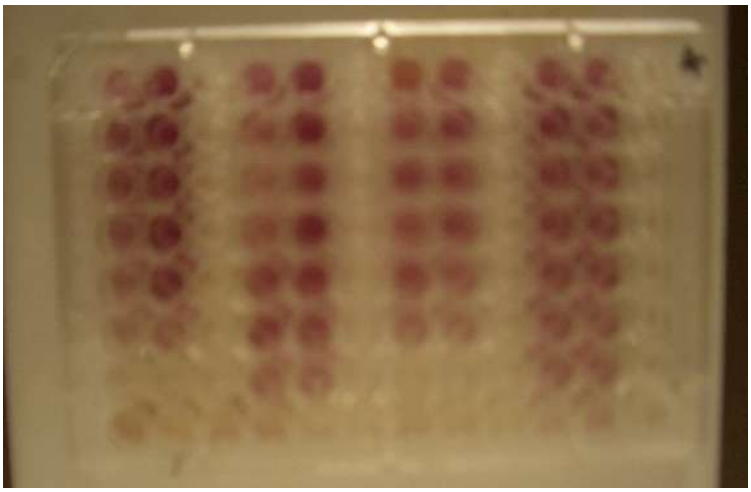


Figure 4. 6: Plate illustrates extracts that are cytotoxic to Vero E6 cell lines.

4.4 ACUTE TOXICITY OF SELECTED PLANT EXTRACTS

Acute toxicity was determined for extracts having low MIC values of ≤ 500µg/disc. There were no deaths or signs of toxicity observed after oral administration of extracts up to 5000mg/kg body weight for extracts of leaves of *H. abyssinica* and *F. africana* tested. However, for the water extract of the stem bark of *H. abyssinica*, mortality was observed with one mice dying within 24 hours. This translated to 20% mortality at 5000 mg/kg body weight of mice. Hence in all extracts tested, LD_{50} was not within the highest concentration tested, 5000mg/kg body weight. Results are summarized in Table 4.6. The surviving mice in the test group gained weight in a similar manner compared to the control, surviving to day 14 (Table 4.7).

Table 4. 7: Acute toxicity results of selected plant extracts on Swiss mice

Plant species	Extract	Concentration	Survivors	% Mortality
Negative control	Distilled water	10% Tween 80	5/5	0
H. abyssinica (L)	Hexane	2500mg/kg	5/5	0
		5000mg/kg	5/5	0
	Dichloromethane	2500mg/kg	5/5	0
		5000mg/kg	5/5	0
H.abyssinica (SB)	Water	2500mg/kg	5/5	0
		5000mg/kg	4/5	20
F. africana (A)	Hexane	2500mg/kg	5/5	0
		5000mg/kg	5/5	0
	Dichloromethane	2500mg/kg	5/5	0
		5000mg/kg	5/5	0

Table 4. 8: Effect of body weight of mice after oral administration of extracts

Plant/part	Extract	Concentration	Mean weight ± S.D.	
			Day $_0$	Day $_{14}$
Control	Distilled water	10% Tween 80	18.8 ± 1.3	21.7 ± 0.1
H. abyssinica (L)	Hexane	2500mg/kg	19.30 ± 1.34	21.8 ± 2.2
		5000mg/kg	17.4 ± 0.89	20.4 ± 1.7
	Dichloromethane	2500mg/kg	18.4 ± 1.71	21.27 ± 0.5
		5000mg/kg	17.6 ± 0.89	20.8 ± 0.6
H.abyssinica (SB)	Water	2500mg/kg	18.6 ± 2.07	21.9 ± 1.8
		5000mg/kg	17.8 ± 0.7	21.8 ± 1.1
F. africana (A)	Hexane	2500mg/kg	18.3 ± 0.44	20.2 ± 1.3
		5000mg/kg	18.9 ± 1.65	21.0 ± 1.9
	Dichloromethane	2500mg/kg	18.0 ± 0.61	20.5 ± 0.2
		5000mg/kg	18.2 ± 0.1	21.1 ± 0.8

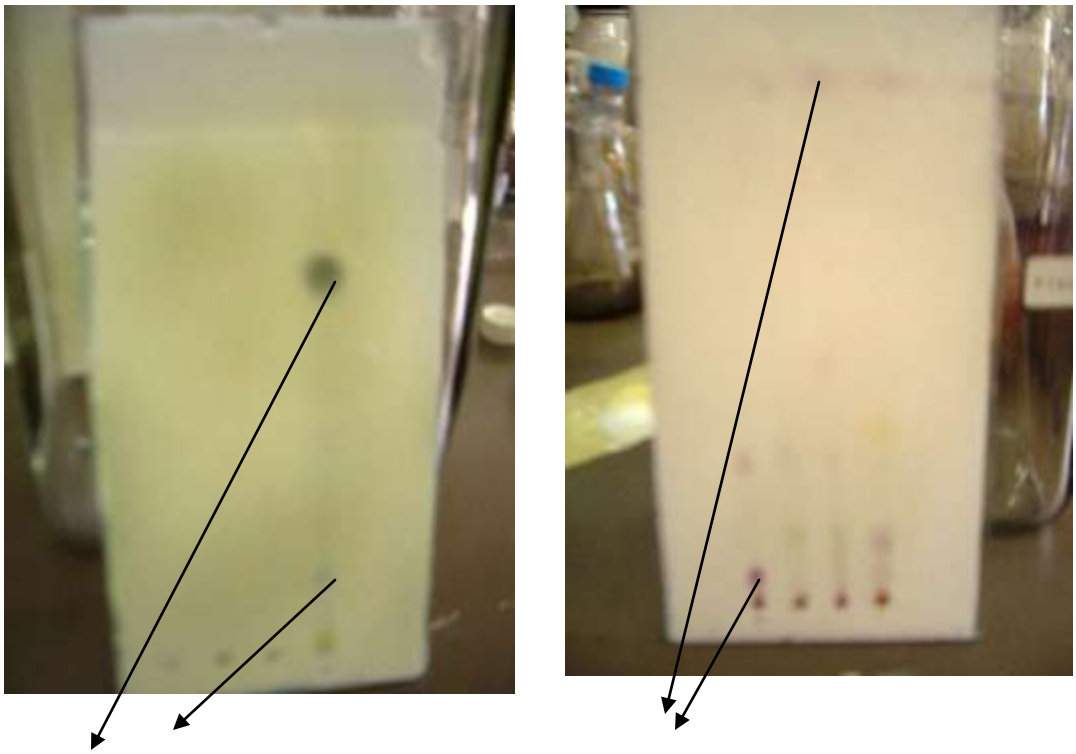
4.5 PHYTOCHEMICAL SCREENING OF TEST PLANTS

Preliminary screening for phytochemicals in the plant extracts revealed presence of terpenoids and phenolics in most plants with anthraquinones present only in hexane extract of leaves of *Hagenia abyssinica*. Flavones and alkaloids were absent in all plant extracts tested (Table 4.8). Hexane extract of leaves of *Hagenia abyssinica* had the most phytochemicals. Terpenoids were present in almost all extracts tested, but were most abundant in *H. abyssinica* extracts while phenolic compounds were present in trace amounts. Photos of TLC plates showing presence of terpenoids and phenolics are found in figure 4.6.

Table 4. 9: Phytochemical profile of selected plant extracts

Plant	Extract	Terpenoids	Alkaloids	Flavones	Phenolics	Anthraquinones
H. abyssinica (SB)	Hexane	++	-	-	+	-
	DCM	++	-	-	-	-
	Methanol	+	-	-	+	-
H. abyssinica (L)	Hexane	++	-	-	+	+
	DCM	+	-	-	-	-
	Methanol	+	-	-	+	-
E. capensis (SB)	Methanol	-	-	-	+	-
F. Africana (A)	Hexane	+	-	-	-	-
	DCM	+	-	-	-	-
A. racemosus(R)	Methanol	-	-	-	+	-

Key ++ Abundant , + Trace, - Not detected.



Blue color phenolic compounds. Purple color terpenoid compounds.

Figure 4. 7: TLC plates positive for phenolics and terpenoids.

CHAPTER FIVE

5.0 DISCUSSION

5.1 EXTRACTION AND PHYTOCHEMICAL SCREENING OF PLANT MATERIALS

On comparing the yields of plant material on the different extraction solvents, the highest yields were observed on water extracts while the lowest on hexane extracts. Non polar compounds were extracted using hexane, while polar compounds were extracted using methanol and water (Harborne, 1984). Hence, suggesting that the selected plants had abundant polar compounds compared to non- polar compounds.

Phytochemical screening of the test plant extracts using TLC visualization reagents revealed presence of terpenoids, phenolics and anthraquinones, which are known to have antimicrobial activity.

Phenolic compounds serve in plant defense mechanism to counteract reactive oxygen species (ROS) in order to survive and prevent molecular damage by microorganisms, insects and herbivores (Sengul *et al*., 2009). Examples of known phenolic compounds with antimicrobial activity include benzoic acid, caffeic acid, cinnamic acid and gallic

acid (Aljadi and Yusoff, 2003). Phenolic compounds were present in 60% of the extracts tested for phytochemicals.

Anthraquinone compounds are known for their antimicrobial activity with emodin and chrysophanol as examples (Ayo *et al*., 2007, Garcia-sosa *et al*., 2006), while drimanes are terpenes which are potent antimicrobials (Gershenzon and Dudareva, 2007). The antimicrobial activity observed in some of the tested plant extracts can therefore be attributed to the presence of these compounds in the plants used.

5.2 ANTIMICROBIAL ACTIVITY AND TOXICITY OF PLANT EXTRACTS

The study indicated a considerable difference in antimicrobial activity between extracts obtained with different solvents. Hexane and dichloromethane extracts were more active than other extracts against Gram- positive bacteria and only one Gram- negative bacteria (*P. aeruginosa*). No activity was observed against *K. pneumoniae* and *E. coli*, both Gram-negative bacteria. The reason for the difference in activity between Gram-positive and Gram- negative bacteria possibly lies in their morphological differences. The Gram- negative bacteria have an outer phospholipid membrane making their cell wall impermeable to lipophilic solutes. On the other hand, the Gram-positive bacteria lack this membrane and are thus more permeable (Nostro *et al*., 2000).

Methanol extracts were able to inhibit both bacteria and fungi with little or no activity was observed on all water extracts. Methanol and water extract polar compounds. However, methanol extracts had better activity compared to water extracts. This may be due to the fact that methanol is able to extract twice as much polar compounds than water (Lapornik *et al.*, 2005).

Most extracts were able to inhibit *P. aeruginosa*, which is among the major pathogens found in hospitals that have rapidly acquired resistance to multiple antibiotics in recent years (Hancock and Speert, 2000). *P. aeruginosa* is known to be more difficult to inhibit and is less susceptible to most antibiotics, hence it's noteworthy that crude extracts demonstrated activity. This may be due to the fact that crude extracts are a mixture of many compounds which in this instance may be working synergistically, hence the demonstrated activity.

This is the first report on the antimicrobial activity of the leaves and stem bark of *H. abyssinica*, a plant commonly found in high altitude areas in East Africa. The antibacterial activity observed in this study could be attributed to the presence of phenolics, terpenoids and anthraquinones found in the plant. In the study, the leaves were more potent antibacterials compared to the stem bark, having high inhibition zones of 20 mm against *S. aureus* and 19.5 mm against MRSA which was higher than

Chloramphenical, whilst exhibiting low MIC values with a range of <1.95 – 3.91µg/disc. This is a significant finding owing to the problem associated with MRSA treatment. MRSA strains are implicated in serious infections and they show resistance to a wide range of antibiotics, limiting treatment options to very few agents such as Vancomycin and Teicoplanin (Baddour *et al*., 2006). However, Vancomycin resistant strains of *S. aureus* have been isolated, underscoring the need for discovery of new antimicrobial agents (Chang *et al*., 2003).

Hexane and dichloromethane extracts of leaves of *H. abyssinica* were highly cytotoxic with IC_{50} values of 7.84 µg/ml and 7.89 µg/ml respectively while the water extract of the stem bark was moderately cytotoxic (31.56 µg/ml). Previous studies have reported presence of phloroglucinols (ά-kosin, kosotoxin and protokosin) in the flowers, which exhibited cytotoxic activity *in vitro* and *in vivo* against MAC tumour cells (Woldemariam *et al*. 1992). The antibacterial activity and cytotoxicity observed in this study may be attributed to presence of kosins which could be in high quantity in the leaves compared to the stem bark. The female flower of the plant has been previously investigated and found to have trypanocidal activity (Nibrent and Wink, 2010).

The dichloromethane extract of *F. africana* was active against *S. aureus* and MRSA with MIC of 31.25µg/disc, but the extract was cytotoxic (IC_{50} 7.91µg/ml). Ferruginol

is a compound that has been previously isolated from this plant and is known for its cytotoxic activity (Koch *et al*., 2006). Ferruginol is a diterpene compound of the abietane class of compounds (Son *et al*., 2005). The active extracts of *F. africana* were found to contain terpenoids, hence ferruginol may have been the compound responsible for the cytotoxicity observed in this study.

Antibacterial compounds either kill the cells (bactericidal) or inhibit its growth (bacteriostatic). The cytotoxicity observed in the most active extracts in this study could influence the antibacterial activity as cytotoxic compounds destroy living cells either selectively or indiscriminately (Kigondu *et al*., 2009). However, toxicity observed either *in vitro* or *in vivo* in a drug candidate is not a basis for disqualification as structural modifications can be made on the compounds in order to synthesize new drug analogues to improve the safety profile (Phillipson, 2007). Examples include Podophyllotoxin from *Podophyllum* species which was too toxic for clinical use. However chemical modifications yielded semi-synthetic analogues Etoposide and Teniposide which are safer and more soluble drugs used for cancer treatments (Kinghorn and Balandrin, 1993).

The methanol extract of *A. racemosus* was active against *P. aeruginosa* (MIC <1.95 µg/disc). However it displayed weak activity against fungi tested (MIC 500-1000

µg/disc), with no activity on all *Candida* spp tested. This differs from previously reported anticandidal activity of the extract by Uma *et al.,* 2009. The difference in the results of both studies may be explained by the fact that the plant samples were collected in two different regions, India and Kenya. The activity of a plant varies with the region it's collected. It is a known fact that plants grown in different climatic regions contain different chemical compositions of active principles (Gilani and Atta-ur-Rahman, 2005). Another possible explanation could be that different strains were tested as some may be resistant isolates (Baddour *et al*., 2006).

The water and methanol extracts of *E. capensis* were found to have weak antibacterial and antifungal activity (MIC 250- 1000 µg/disc). Similar results were reported by Rabe and Van staden, 1997. Reasons for the high MIC values could be that the extracts tested which are still in impure form, may contain compounds that act antagonistically or that the active compound/ s are present in very low concentrations in the extracts. In an earlier study, the bark of *E. capensis* has been reported to be active against drug resistant strains of *M. tuberculosis* (Lall and Meyer, 1999).

There was little antimicrobial activity observed for all water extracts tested in this study. Medicinal plants are traditionally taken as aqueous extracts (Gachathi, 1989; Kokwaro, 1993), and in combination with others at very high doses (Azas *et al*., 2004). This may

explain why low activity was observed for the aqueous extracts, when tested singly at low concentrations (1000 µg/disc).

In recent years there is increasing trend for using alternative system of medicine. It is argued, that such drugs are not only effective but also very safe as compared to allopathic drugs for the similar indications. The claim that natural plant products are safe should be accepted only after the plant product passes through toxicity testing using modern scientific methods.

In the study, the hexane and dichloromethane extracts of the leaves of *H. abyssinica*, and the dichloromethane extract of *F. africana* were found to be cytotoxic. However, these extracts did not demonstrate acute toxicity *in vivo*. The difference can be explained in the function of cells *in vivo*. An important function of cells *in vivo* is their ability to metabolize chemicals to more- or less- toxic compounds. This function is usually expressed to a small extent in cultured cell, which results in limited activation or deactivation of test chemicals or in the *in vitro* accumulation of intermediates that do not occur *in vivo* (Walum, 1998)

Acute toxicity studies in animals are of value in predicting potential toxic effects of a chemical in human beings exposed to near fatal doses. From these studies the nature of

acute response in man as a result of exposure to these phytochemicals may be anticipated (Jaykaran *et al*., 2009).

5.3 CONCLUSIONS

The present study established the antibacterial activity of leaves of *H. abyssinica* against *S. aureus* and MRSA as the plant had the highest activity observed. The activity against *S. aureus*, MRSA and *P. aeruginosa* is worth noting as extracts had a higher or equal activity compared to the standard drug. This demonstrates the potential of the plant as a source of new antimicrobial agents that can be effective for treatment of infections arising from the three pathogens.

The observation that *S. aureus*, MRSA and *P. aeruginosa* were susceptible to plant extracts at minimum inhibitory concentrations of <1.95µg/disc strongly suggests that leaves of *H. abyssinica* and roots of *A. racemosus* may be effective in the treatment of infections caused by these pathogens.

With the exception of *E. capensis*, none of the other water extracts demonstrated antibacterial activity. This indicates that the extracts are deficient in compounds that have antibacterial activity against bacteria used in this study.

Most extracts did not exhibit activity against the fungal strains used. Methanol extract of *A. racemosus* demonstrated weak activity against *C. neoformans, T. mentagrophytes* and *M. gypseum*, while the methanol extract *of E. capensis* also displayed weak activity against *T. mentagrophytes*. Extracts of *H. abyssinica* stem bark, leaves, and *F. africana* had no antifungal activity. This indicates that the plants in the study might be exploited for use against the fungal microorganisms in this study.

In vitro cytotoxicity was observed in extracts that had shown antimicrobial activity with 36% of extracts tested being cytotoxic having IC_{50} < 90µg/ml. However, this did not translate into *in- vivo* acute toxicity as all extracts tested LD_{50} was not within the highest concentration tested of 5000mg. We can therefore conclude that *in vitro* cytotoxicity does not translate directly to *in vivo* acute toxicity probably due to biotransformation of the constituents into less toxic metabolites in the body.

Phytochemical studies of the plants selected have shown presence of phenolics, terpenoids and anthraquinones in some of the plants considered. These are thought to be responsible for the antimicrobial activity and cytotoxicity observed in some of the extracts.

5.4 RECOMMENDATIONS

1) The study validates the ethnobotanical use of the selected plants, more so *Hagenia abyssinica* which had very good antibacterial activity. It is recommended that bioassay guided fractionation on the hexane and dichloromethane extracts of *H. abyssinica* leaves should be carried out in order to isolate and identify the active principles responsible for the activity observed in this plant.

2) Chronic toxicity should be carried out on the crude extracts to establish their safety profiles. This will aid in calculating its safety dosage and informing its long term use as a traditional medicine.

3) The search for new antimicrobial agents is continuous as current drugs have limitations. Medicinal plants are one of the sources, hence sustainable use and conservation of these plants is recommended.

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APPENDICES

Appendix 1: Data sheet for antibacterial assay for crude extracts

Treatment	Bacterial strains and inhibition zone diameter of extracts.																
	S. aureus				MRSA				*P. aeruginosa*				*K. pneumoniae*			*E. coli*	
Chloramphenical	17	18	17		15	15	15	15	16	17	17	16	30	30	29	18	18
HASB- HEX	8	10	-		10	12	8		-	-	-		-	-	-	-	-
HASB- DCM	8	9	9		8	10	10	8	-	-	-		-	-	-	-	-
HASB- METH	12	10	11	11	10	11	11	11	8	16	11	14	-	-	-	-	-
HASB- WAT	-	-	-		-	-	-		10	12	12		8	8	8	-	-
HAL- HEX	18	16	16		18	22	18		15	12	12		-	-	-	-	-
HAL- DCM	22	20	18		22	22	16	18	14	17	-		-	-	-	-	-
HAL- METH	8	7	8	8	8	7	8	7.5	14	12	10	12	-	-	-	-	-
HAL – WAT	-	-	-		-	-	-		-	-	-		-	-	-	-	-
FAA – HEX	10	9	9		9	8	11		10	7	-		-	-	-	-	-
FAA- DCM	10	10	11		11	10	10	11	10	9	10		-	-	-	-	-
FAA –METH	7	6.5	-		-	-	-		9	6.5	-		-	-	-	-	-
FAA- WAT	-	-	-		-	-	-		7	7	-		-	-	-	-	-

Apendix 1: Continued

Treatment	Bacterial strains and inhibition zone diameter of extracts.																
	Staphylococcus aureus				MRSA				*P. aeruginosa*				*K. pneumoniae*			*E. coli*	
ECSB- HEX	-	-	-		-	-	-		-	-	-		-	-	-	-	-
ECSB- DCM	7	8	7		-	-	-		-	-	-		-	-	-	-	-
ECSB- METH	12	10	10	10	10	14	11	11	8	12	12		-	-	-	-	-
ECSB- WAT	-	-	-		10	11	9	7	6.5	-	-		-	-	-	-	-
ARR- HEX	-	-	-		-	-	-		-	-	-		-	-	-	-	-
ARR- DCM	-	-	-		-	-	-		12	11	-		7	7	6.5	-	-
ARR- METH	-	-	-		-	-	-		12	11	12		-	-	-	-	-
ARR- WAT	-	-	-		-	-	-		-	-	-		7	7	-	-	-

Key:

- denotes no inhibition zones observed

HASB- *Hagenia abyssinica* (Stem bark)

HAL- *Hagenia abyssinica* (Leaves)

FAA- *Fuerstia africana* (Aerial parts)

ECSB- *Ekerbergia capensis* (Stem bark)

ARR – *Asparagus racemosus* (Roots)

HEX- Hexane extract

DCM- Dichloromethane extract

METH- Methanol extract

WAT- Water extract

Appendix 2: Data sheet for antifungal assay for crude extracts

Treatment	Fungal strains and inhibition zone diameter of extracts.														
	C. albicans		*C. parapsilosis*		*C. krusei*			*C. neoformans*		*T. mentagrophytes*			*M. gypseum*		
Miconazole	17	17	17	17	11	10	11	15	15	Nd	Nd	Nd	Nd	Nd	Nd
Fluconazole	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	24	25	24	26	25	24
HASB- HEX	-	-	-	-	-	-	-	-	-	7	7	7	-	-	-
HASB- DCM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HASB- METH	-	-	-	-	-	-	-	-	-	-	-	-	7	8	7
HASB- WAT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HAL- HEX	-	-	-	-	-	-	-	-	-	7	7	6.5	-	-	-
HAL- DCM	-	-	-	-	-	-	-	-	-	8	6.5	7	-	-	-
HAL- METH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HAL – WAT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FAA – HEX	-	-	-	-	-	-	-	-	-	10	6.5	7	-	-	-
FAA- DCM	-	-	-	-	-	-	-	-	-	8	6.5	7	-	-	-
FAA –METH	-	-	-	-	-	-	-	-	-	8	7	7	-	-	-
FAA- WAT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Apendix 2: Continued

Treatment	Fungal strains and inhibition zone diameter of extracts.														
	C. albicans		*C. parapsilosis*		*C. krusei*			*C. neoformans*		*T. mentagrophytes*			*M. gypseum*		
ECSB- HEX	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ECSB- DCM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ECSB- METH	-	-	-	-	-	-	-	-	-	11	12	14	8	7	9
ECSB- WAT	-	-	-	-	-	-	-	-	-	-	-	-	9	7	7
ARR- HEX	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ARR- DCM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ARR- METH	-	-	-	-	7	8	6.5	16	6.6	11	12	14	8	9	7
ARR- WAT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Key:

- denotes no inhibition zones observed

Nd- Not determined

HASB- *Hagenia abyssinica* (Stem bark)

ECSB- *Ekerbergia capensis* (Stem bark)

DCM- Dichloromethane extract

HAL- *Hagenia abyssinica* (Leaves)

ARR – *Asparagus racemosus* (Roots)

METH- Methanol extract

FAA- *Fuerstia africana* (Aerial parts)

HEX- Hexane extract

WAT- Water extract

Appendix 3: Descriptive statistics for *S. aureus* group information

Treatment	Mean	Variance	Standard Deviation	Standard Error
Chloramphenical	17.333	0.333	0.577	0.333
HASB- HEX	9.000	2.000	1.414	1.000
HASB- DCM	8.667	0.333	0.577	0.333
HASB- METH	11.000	0.667	0.816	0.408
HAL- HEX	16.667	1.333	1.155	0.667
HAL- DCM	20.000	4.000	2.000	1.155
HAL- METH	7.750	0.250	0.500	0.250
FAA- HEX	9.333	0.333	0.577	0.333
FAA- DCM	10.667	0.333	0.577	0.333
FAA- METH	6.750	0.125	0.354	0.250
ECSB- DCM	7.500	0.500	0.707	0.500
ECSB- METH	10.500	1.000	1.000	0.500

Appendix 4: Descriptive statistics for MRSA group information

Treatment	Mean	Variance	Standard Deviation	Standard Error
Chloramphenical	15.000	0.00	0.00	0.00
HASB- HEX	9.500	3.667	1.915	0.957
HASB- DCM	9.333	1.333	1.155	0.667
HASB- METH	10.75	0.250	0.500	0.250
HAL- HEX	19.333	5.333	2.309	1.333
HAL- DCM	19.50	9.00	3.00	1.500
HAL- METH	7.625	0.229	0.479	0.239
FAA- HEX	9.333	2.333	1.528	0.832
FAA- DCM	10.50	0.333	0.577	0.289
ECSB- METH	11.50	3.00	1.732	0.866
ECSB- WAT	10.50	0.500	0.707	0.500

Appendix 5: Descriptive statistics for *P.aeruginosa* group information

Treatment	Mean	Variance	Standard Deviation	Standard Error
Chloramphenical	16.50	0.333	0.577	0.289
HASB- METH	12.250	12.250	3.500	1.750
HAL- HEX	13.00	3.00	1.732	1.00
HAL- DCM	15.50	4.50	2.121	1.500
HAL- METH	12.000	2.667	1.633	0.816
FAA- HEX	8.500	4.500	2.121	1.500
FAA- DCM	9.667	0.333	0.577	O.333
FAA- METH	7.750	3.125	1.768	1.250
FAA- WAT	7.00	00	00	00
ARR- METH	11.667	0.333	0.577	0.333
ECSB- METH	10.667	5.333	2.309	1.333
ECSB- WAT	7.500	1.750	1.323	0.764

Appendix 6: Descriptive statistics for *K. pneumoniae* group information.

Treatment	Mean	Variance	Standard Deviation	Standard Error
Chloramphenical	29.667	0.333	0.577	0.333
ARR- DCM	6.833	0.083	2.89	0.167
ARR- WAT	7.00	00	00	00

Appendix 7: Descriptive statistics for *C. krusei and C. neoformans* group information

Microorganism	Treatment	Mean	Variance	Standard Deviation	Standard Error
C. krusei	Miconazole	10.667	0.333	0.577	0.333
	ARR- METH	7.375	0.563	0.750	0.375
C. neoformans	Miconazole	15.000	00	00	00
	ARR- METH	11.250	45.125	6.718	4.750

Appendix 8: Descriptive statistics for *T. mentagrophytes* group information

Treatment	Mean	Variance	Standard Deviation	Standard Error
Fluconazole	24.33	0.333	0.577	0.333
HASB- HEX	7.00	0.00	0.00	0.00
HAL- HEX	6.833	0.083	0.289	0.167
HAL- DCM	7.167	0.583	0.764	0.441
FAA- HEX	7.833	3.583	1.893	1.093
FAA- DCM	7.167	0.583	0.764	0.441
FAA- METH	7.333	0.333	0.577	0.333
ECSB- METH	12.333	2.333	1.528	0.882
ARR- METH	12.333	2.333	1.528	0.882

Appendix 9: Descriptive statistics for *M. gypseum* group information

Treatment	Mean	Variance	Standard Deviation	Standard Error
Fluconazole	25.00	1.00	1.00	0.577
HASB- METH	7.333	0.333	0.577	0.333
ECSB- METH	8.00	1.00	1.00	0.577
ECSB- WAT	7.667	1.333	1.155	0.667
ARR- METH	10.667	1.00	1.00	0.577