Antimicrobial Effects of Selected Herbal Extracts on Clinical Multi-Drug Resistant Gram-Negative Bacteria

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DECLARATION

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

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DEDICATION

This work is dedicated to my wife and best friend Edna Muthamia, my parents Mr. and Mrs. Njire, my brothers and sisters. Thank you for all the support you have given me. Dad and Mum you have laid in me a good foundation that has seen me through to this level of education. May God forever bless you. You are the very best.

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LIST OF ABBREVIATIONS

AST	Antimicrobial Susceptibility Test
ATCC	American Type Culture Collection
bp	Base Pairs
CFU	Colony Forming Units
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KEMRI	Kenya Medical Research Institute
MDR	Multidrug Resistant Bacteria
MGEs	Mobile Genetic Elements
MHA	Muller-Hinton agar
MICs	Minimum Inhibitory Concentrations
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
Rpm	Revolutions Per Minute
SE	Standard Error
SLC	Sub-Lethal Concentration
TBE	Tris-Borate Ethylenediaminetetraacetic Buffer
VCR	Variable Cassette Region
WHO	World Health Organization

ABSTRACT

The rise in antibiotic resistance has resulted in decreasing numbers of effective antimicrobial agents available to treat infections caused by multi-drug resistant (MDR) bacteria. This has necessitated a search for new antimicrobial agents. Herbal remedies may offer alternative treatment options especially because they elicit little or no transferable resistance if used in optimal concentrations. This study evaluated antimicrobial properties of 10 plants traditionally used as herbal remedies against a total of 27 MDR Gram-negative bacterial isolates. The extracts were obtained through organic (methanol) and inorganic (water) solvents extraction. Susceptibility of the test strains to conventional antibiotics was determined by the disc diffusion technique while molecular characterization of resistance was done through plasmid profiling and PCR screening for the presence of Class 1 integrons and genes encoding β-lactamases such as *bla-*$_{SHV}$ and *bla-*$_{TEM.}$ Determination of the Minimum Inhibitory Concentrations (MICs) and the sub-lethal concentrations of the effective extracts was done by broth cultures followed by colony counts. The test isolates were habituated in sub-lethal extract concentrations for 72 h to investigate effect on their sensitivity to conventional antibiotics. Mating experiments were employed in order to determine if the extracts facilitate or inhibit conjugative transfer of resistance markers. Out of the 27 MDR strains, 74% contained class 1 integrons while 80% were positive for the *bla-*$_{SHV}$ and *bla-*$_{TEM}$ genes. Out of the 10 plants, only *Warbugia ugandensis* root and stem-bark extracts were active against the MDR strains and their inhibitory effect was significantly higher than that of other plant extracts (t-test, $p<0.001$). Methanol extracts from the root and stem-bark of this plant were inhibitory but not lytic against test strains with an MIC of 42 µg/ml. The

inhibitory effects of the root or stem-bark extracts was significantly reduced when mixed with the leaf extracts (t-test, $p< 0.02$). Susceptibility of test strains to conventional antibiotics was not significantly affected before and after exposure to sub-lethal extract concentration (t-test, $p>0.005$). The extracts did not stimulate or inhibit conjugal transfer of resistance determinants. Methanol extracts from the root and stem-bark of *W. ugandensis* may provide potential sources for further development of alternative antimicrobial agents that may find use in the treatment of MDR infections.

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Misuse of antibiotics over the years has resulted in the emergence of resistant bacterial strains. Antimicrobial resistance is a natural biological phenomenon of response to the selective pressure of an antimicrobial agent. This resistance is due to point mutations in the chromosomes or due to acquisition of extra-chromosomal DNA (plasmids) or other mobile genetic elements such as transposons and integrons (Rodrıguez *et al.,* 2006). Some strains belonging to the family Enterobacteriaceae and *Pseudomonas* sp that cause diarrhoea, urinary tract infections, and sepsis, are resistant to virtually all classes of the older antibiotics (Falagas and Bliziotis, 2007).

A bacterium is said to be multidrug resistant if it exhibits resistance to more than three classes of antibiotics which have different modes of action (Koronakis *et al.,* 2000). The emergence of multidrug resistant strains of Gram-negative bacteria especially among infectious species of *Pseudomonas, Klebsiella, Enterobacter, Acinetobacter* and *Salmonella* is worrisome in the present therapeutic scenario. Such infectious species are not only resistant to first line of treatments, but also to the more expensive second and third-lines of treatment. MDR strains impact on the quality of healthcare since they contribute to unfavourable clinical outcomes, strain the utilization of hospital resources and increase the burden of effective infection control (Blot *et al.,* 2007). MDR strains also lead to increased morbidity and mortality due to reduced effectiveness of antimicrobial treatment (Alam *et al*., 2008).

The extensive use of antimicrobials in the community and hospital settings has led to increased prevalence of MDR strains. This is due to genetic changes among isolates which leads to loss of potency of antibiotics and antiseptics (Hamilton-Miller, 2004). This has lead to the re-emergence of infections that were once much easier to manage like Tuberculosis and Cholera as well as emergence of new types of infections such as leptospirosis (Bielaszewska and Karch, 2004). This scenario has necessitated a search for new antimicrobial agents that present alternative mechanisms of inhibition of microbial growth while reducing chances of the bacteria to develop genetically encoded resistance. Plants offer the most promising sources of such alternative antimicrobials (Morens *et al*., 2004).

Most plant products with antimicrobial effect are usually more active against Gram-positive bacteria such as *Clavibacter* sp but are less active against the Gram-negative bacteria like *Pseudomonas* sp (Suffredini *et al.,* 2006). This suggests that the fundamental morphological differences in the cell wall and membrane organization of Gram negative and Gram-positive organisms modulate their susceptibility to plant-derived products. Gram-negative bacteria possess an outer phospholipidic membrane with structural lipopolysaccharide components which are not found in Gram-positive bacteria. One possible explanation is that the cell wall composition makes the bacteria impermeable to lipophilic solutes, and the porins in the cell wall do not allow the penetration of high molecular mass hydrophilic solutes present in some herbal extracts. In view of this, identification of effective herbal extracts against Gram-negative bacteria would highly validate their potential as alternative broad-spectrum antimicrobials.

1.2 Risk factors associated with acquisition and spread of multi-drug resistance strains

The most important factor influencing the emergence of antibiotic-resistant bacterial infections has been the extensive use of antimicrobial agents within hospitals and in the community settings as well as for prophylaxis and as growth-promoting agents in animal husbandry (Shea, 2004). Inappropriate use mainly results from self medication, available and affordable antibiotics dispensed over the counters of pharmacists, non-investigational based prescription by health providers, poor quality assurance and quality control of the recommended dosage on available drugs as well as wrong diagnosis of infections (Bari *et al.,* 2008). The resultant antibiotic pressure selects for highly resistant bacterial strains.

Patients infected with MDR strains are more likely to develop therapeutic failure, experience longer duration of hospital stay and may require treatment with more expensive broad-spectrum antibiotics (Foster and Grundmann, 2006). Longer hospital stays may increase the chances of nosocomial infection to the patient besides increasing chances of his/her own commensals picking antibiotic resistance genes through horizontal gene transfer (Raymond *et al.,* 2002). During such times, patients are often treated with broad-spectrum antibiotics, which kill their normal gut flora while selecting for resistant strains. Such MDR nosocomial strains are expensive to control and extremely difficult to eradicate (Bari *et al.,* 2008). Staff compliance with basic infection control practices such as hand washing is often inadequate, and the shortage of health-care staff and isolation facilities make the control of the spread of MDR strains difficult. A combination of these factors lead to emergence of MDR

strains which present a major crisis in clinical settings, especially in countries with limited therapeutic options.

1.3 Mechanisms of antibiotic resistance

Antibiotic resistance can be 'intrinsic', or 'acquired' (Anadon *et al*.**,** 2005). Intrinsic or 'natural' resistance is inherent to a bacterial species and may involve the absence of the drug target or the presence of low-affinity drug targets, or low membrane permeability of the drug, inactivation of the antibiotics and the presence of drug efflux mechanisms. Such pumps that have been described among *E. coli, Salmonella,* and *Pseudomonas* spps flush the antimicrobial agent out of the cell before lethal concentrations are reached (Vila *et al*., 2007). Bacteria resist antibiotics containing a β-lactam ring such as penicillins and cephalosporins through hydrolysis (Mascaretti, 2003). Another strategy for resistance is the step-wise alteration in the primary site of action of the antibiotic through acquisition of point mutations in the encoding gene (Todd *et al*., 2007). Such bacteria continue to produce the original target that is sensitive to the agent but also produce alternative penicillin binding proteins, (PBP2a), that are not inhibited by antibiotics (Davies *et al*., 2007).

1.4 Transmission of antibiotic resistance

The acquisition of antibiotic resistance phenotype occurs via the mutation of pre-existing genes or by horizontal transmission of plasmids carried on mobile genetic elements (MGEs) (Normark, 2002). The mobile genetic elements mostly implicated in the dissemination of antibiotic resistance genes are conjugative resistance

plasmids (R-plasmids) and transposons (Johnsborg, 2007). Another class of elements known as integrons have also been implicated in the dissemination of antibiotics resistance (Nogrady *et al*. 2006) and harbour cassettes encoding antibiotic resistance genes. The integrons may in turn be harboured on chromosomes or in plasmids thereby increasing their chances of dissemination. Among the nine classes of integrons that have been identified, class 1, 2 and 3 are the highly involved in the mobilization of antibiotic resistance markers and integron class 1 are the most prevalent among clinical isolates such *E. coli, Salmonella, Shigella, Klebsiella* and *Pseudomonas* (Gu *et al.*, 2007).

Class 1 integrons have two conserved segments, the 5' conserved segment (5'-CS) and the 3' conserved segment (3'-CS) that flank the variable region which contains gene cassettes encoding antibiotic resistance determinants. The 5'-CS contains the *intI1* gene, which encodes the type 1 integrase responsible for site-specific insertion and excision of the gene cassettes. Other genes found at the 5'-CS include the promoter responsible for transcription of the inserted cassettes. The *attI1* site is responsible for recombination, that is, insertion of the cassettes into the integron framework. The 3'-CS contains the *qacEΔ1* and *sul1* genes, which encode resistance to quaternary ammonium compounds and to sulfonamides respectively. The 3'-CS is normally found after the 5'-CS. However, class 1 integrons do not always contain the 3'-CS. The ability of class 1 integrons to integrate diverse genes and their inherent mobility has led to their pre-eminent role in the spread of resistance determinants in humans (Nogrady *et al*., 2006). None of these mobile genetic elements have been implicated in the emergence and dissemination of antibiotic resistance.

1.5 Alternative Remedies

1.5.1 Need for new antimicrobials

Owing to a battery of resistance mechanisms to antibiotics that bacteria mount, it is evident that the prospects for development of new, effective, affordable and safe conventional antibiotics with activity against MDR organisms look grim (Boucher *et al*., 2009). Furthermore, the probability that such a discovery would lead to commercial production remains low. This is because the conventional process of drug discovery has several distinct and expensive stages. This includes, acquisition of the source material, extraction of the active compounds, primary screening against a range of pathogens, isolation and chemical characterization of the active compounds. Other processes involve assaying the compounds in tissue cultures and experimental animals, elucidation of the active ingredients, structural chemistry and preclinical production for animal and human trials, clinical approval and finally marketing and distribution.

Natural products from microorganisms have been the primary source of antimicrobials, but with the increasing acceptance of herbs as an alternative source, the screening of plants for active compounds has become important because these may serve as promising sources of novel agents (Koduru *et al*., 2006). Furthermore, the pharmacological interests in the efficacy and safety of herbal remedies have grown in recent years because of the realization that many people use these agents for self medication. For example, in the developing world, many patients purchase herbal preparations for management of various ailments some of which include

bacterial infections (Anon, 2002). According to the WHO, medicinal plants would be the best source for a variety of drugs (Nair and Chanda, 2006). There is however limited information on the pharmacology and toxicology of the herbal remedies. Thus, *in vitro* screening methods could provide the needed preliminary data to select crude plant extracts with potentially useful properties for further chemical and pharmacological investigations (Koduru *et al.,* 2006).

1.5.2 Diversity and utilization of plant antimicrobial agents

It is believed that plants make antimicrobial agents to defend themselves from potential pathogens. Some of these agents are preformed while others are activated by the presence of pathogens (Jones and Dangl, 2006). The mechanism of resisting bacterial infection by plants may therefore include reinforcement of the cell wall, biosynthesis of lytic enzymes, production of secondary metabolites and related proteins (Bednarek and Osbourn, 2009).

The plant compounds that have received more attention are the phytoalexins (Figure 1) and phytoanticipins (Figure 2). Phytoalexins are antimicrobial compounds that require *de novo* expression of the enzymes involved in their biosynthetic pathways after elicitation by the presence of the pathogen (Bednarek and Osbourn, 2009). Phytoanticipins on the other hand are low molecular weight antimicrobial compounds that are present in plants before challenge by microorganisms (Pedras *et al*., 2007).

Saponins are glycosylated phytoanticipins that are found in a wide range of plant species and can be divided into three major groups; triterpenoid, steroid or steroidal glycoalkaloid, depending on the structure of their aglycones (Jones and Dangl, 2006). As these agents have potent antimicrobial activities, it is proposed that their natural role in plants is to confer protection against pathogens (Jones and Dangl, 2006).

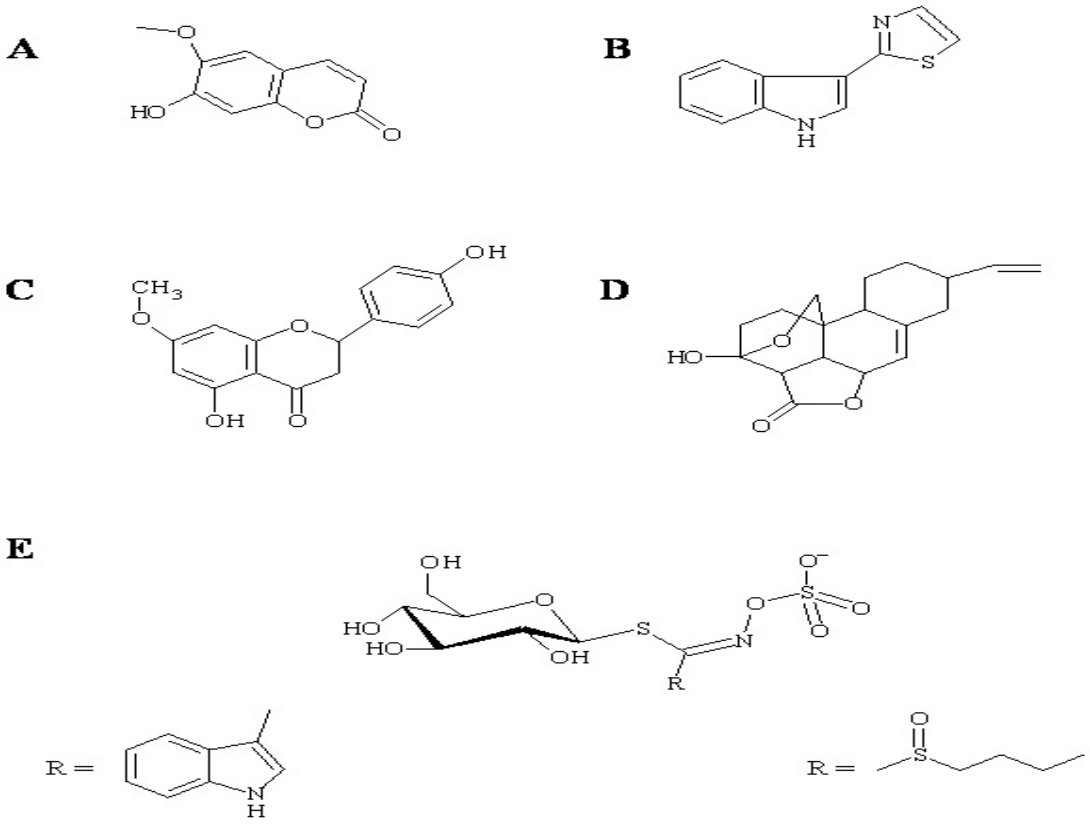

Figure 1. Examples of Arabidopsis phytoalexin structures. (A) Scopoletin from tobacco, (B) camalexin from *A. Thaliana*, (C) sakuranetin, (D) nomilactone B from rice, and (E) glucosinolates from *Brassicacea* (Gonzalez-Lamothe *et al*., 2009).

Another group of important plant antimicrobials are quinones (Figure 3). These compounds have aromatic rings with two ketone substitutions and are known to

complex irreversibly with nucleophilic amino acids in proteins (Bittner, 2006) often leading to loss of protein function. For that reason, the potential range of quinone antimicrobial effects is great. Lenta *et al.* (2007) described two anthraquinones, zenkequinones A and B, from s*tereospermum zenkeri* which were active against multiresistant strains of bacteria, with zenkequinones B showing the high antibacterial activity (MIC 9.50 µg/ml) against gram-negative *Pseudomonas aeruginosa*.

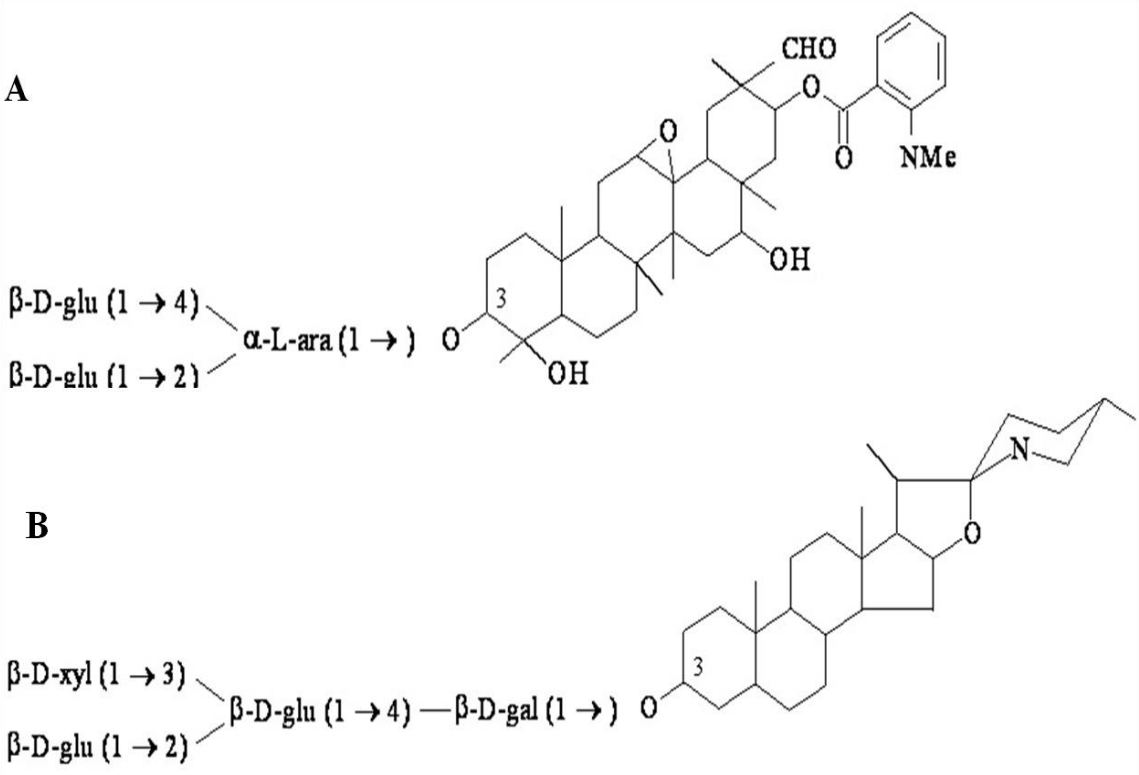

Figure 2. Examples of antimicrobial phytoanticipin structures. (A) The major oat root saponin avenacin A-1, and (B) the saponin α-tomatine from tomato (Gonzalez-Lamothe *et al*., 2009).

Flavonoids are hydroxylated phenolic substances that occur as a C6-C3 unit linked to an aromatic ring (Figure 3). These chemicals are known to be synthesized by plants in response to microbial infection and have been found to be effective antimicrobial substances against a wide array of microorganisms *in vitro* (Jones and Dangl, 2006). Catechins (flavonoids) from green Tea were found to be more bactericidal than conventional antibiotics like tetracycline, at comparable concentrations (Friedman *et al*., 2006).

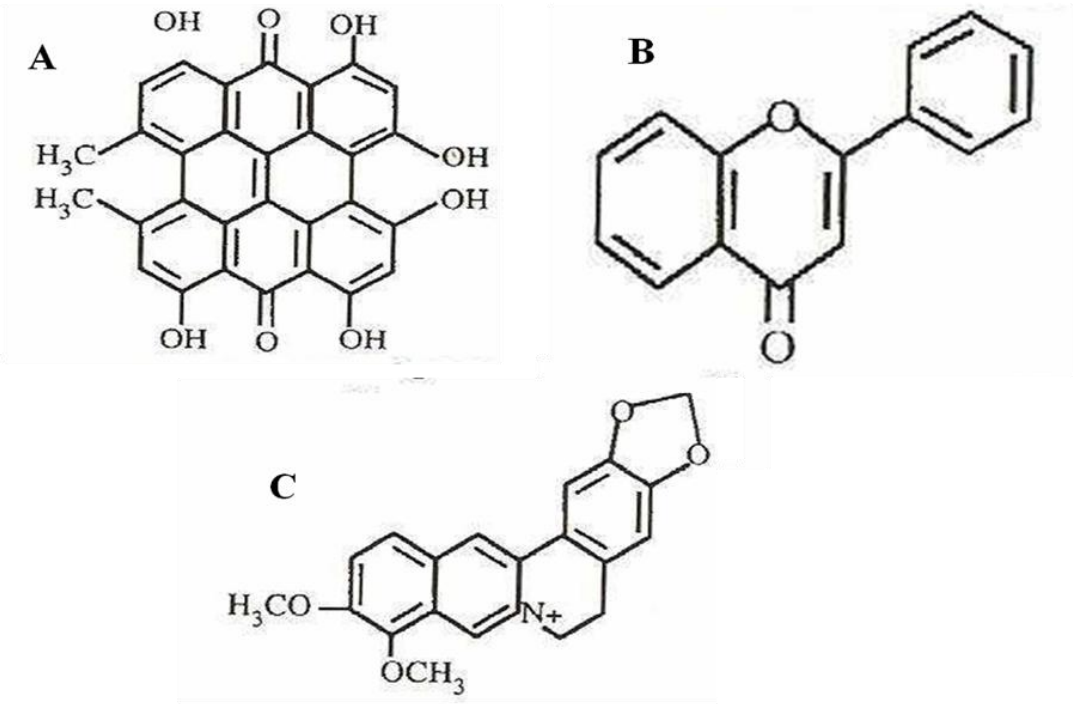

Figure 3. Other examples of plant antimicrobial structures. (A) Hypericin, an example of a quinone, (B) general structure of a flavone, (C) Berberine, an example of an alkaloid (Gonzalez-Lamothe *et al*., 2009).

Heterocyclic nitrogen compounds such as alkaloids (Figure 3) have also been tested against bacteria. Bioassay-guided fractionation of the active ingredients from *Chelidonium majus* led to the isolation of benzo[c]phenanthridine-type alkaloids, 8-hydroxydihydrosanguinarine and 8-hydroxydihydrochelerythrine, which were potently active against Methicillin-resistant *Staphylococcus aureus* strains (MRSA) with MICs/MBCs ranges of 0.49-15.63/1.95-62.50 μg/ml (Zuo *et al.*, 2008).

1.5.3 Use of herbal extracts in treatment of bacterial infections

Plant antimicrobial compounds have diverse chemical structures that work in a synergistic manner (Lewis and Ausubel, 2006). The synergistic action of the multiple classes of antibacterial products, including phenolic acids and polyphenols, phenanthrenes, flavonoids, terpenoids and essential oils improves their efficacy as alternative antimicrobials. Such synergistic reaction mechanisms clear infections more efficiently, resulting in less toxicity to the patient. Efficient inhibition and lysis of bacteria decreases the risk of developing resistance through gradual habituation by the pathogen (Miyasaki *et al*, 2010). Additionally, extracts from medicinal plants have been used in the treatment of infectious diseases due to their availability and affordability (Lee *et al*., 2007). They have been found to be effective against urinary tract infections, gastrointestinal disorders, respiratory diseases and cutaneous infections (Somchit *et al.*, 2003). Antibacterial activity of the essential oils as well as eugenol from *Ocimum gratissimum* have been used for the treatment of pneumonia, diarrhoea and conjunctivitis (Nakamura, 1999). Menthol, from peppermint (*Mentha piperita*) have antibacterial activity against *Clostridium sporogenes*, E*nterobacter*

aerogenes, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* among other pathogens (Schuhmacher *et al*., 2003). *Aloe vera* L. gel has also been used for the treatment of digestive diseases (Langmead *et al.* 2004).

1.5.3 Interplay of antibiotics and plant antimicrobial compounds

Plant products defined as "antibiotic potentiators" could allow the current conventional arsenal of antibiotics to gain back some of the therapeutic applications lost due to spread of MDR strains, while others defined as "virulence attenuators" could assist the host immune system to adequately respond to the pathogen invasion (Figure 4).

1.5.3.1 Plant-derived products as antibiotic potentiators

The MDR pumps are among the major contributors in the intrinsic resistance of bacteria against a variety of toxic molecules such as alkaloid amphipathic cations that may be related to secondary metabolites produced in plants (Pages *et al*., 2008). The bacterial efflux pump inhibitors have demonstrated marked synergy when used in combination with conventional antibiotics against both Gram positive bacteria like *S. aureus* and Gram negative bacteria like *P. aeruginosa*. Therefore, plant products such as essential oils, terpenoids and sesquiterpenes can be considered antibiotic potentiators. Among putative efflux pump inhibitors, a catechin (epigallocatechin-gallate) found in green tea extracts abolished tetracycline resistance in staphylococcal isolates expressing TetK, one of the efflux pumps found in Gram positive bacteria (Roccaro *et al.*, 2005).

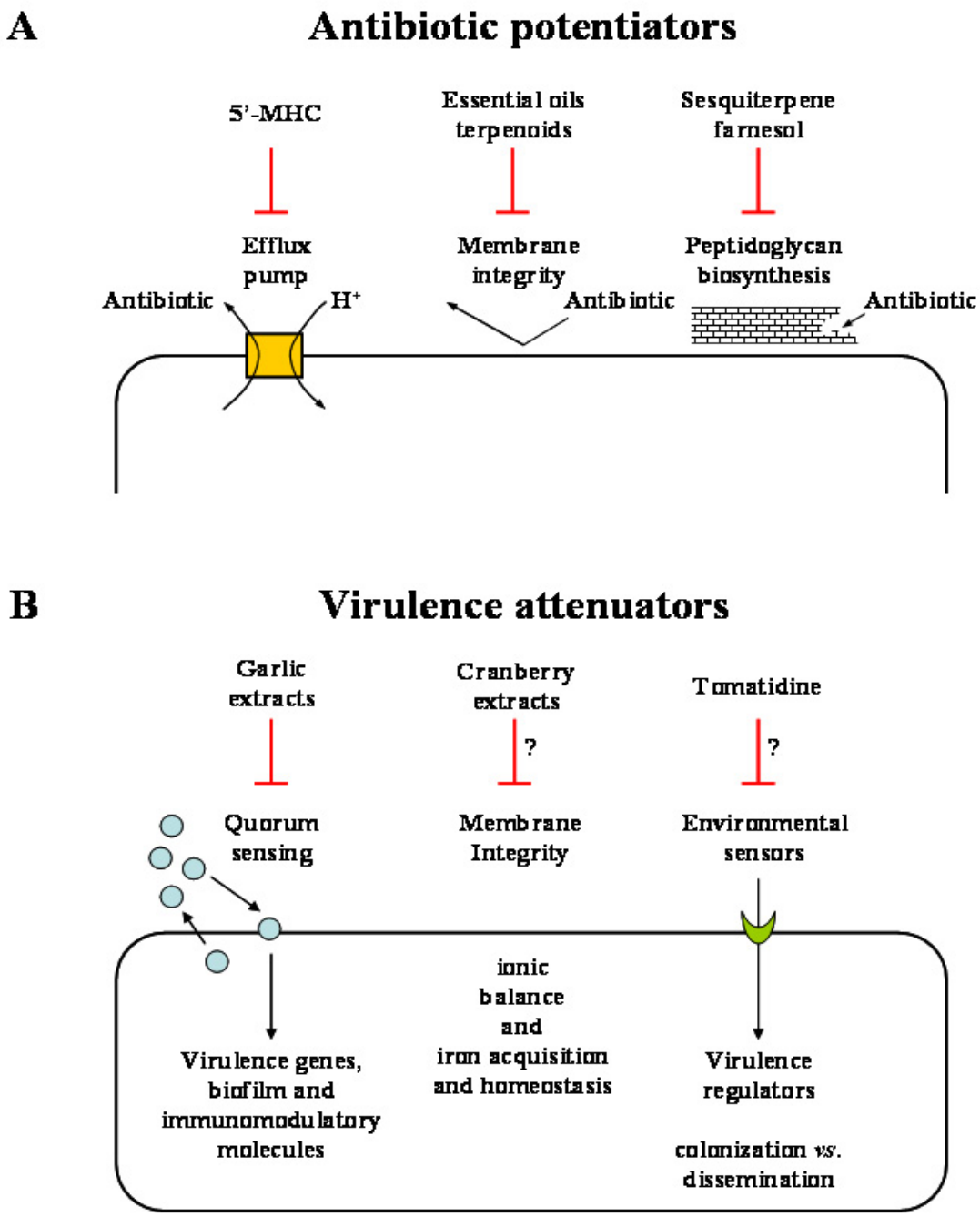

Figure 4. Examples of plant "antibiotic potentiators" (A) or "virulence attenuators" (B). 5'-MHC: 5'-methoxyhydnocarpin (Gonzalez-Lamothe *et al*., 2009).

The medicinal plants *Berberis* spp were shown to produce both the antibacterial alkaloid berberine as well as the norfloxacin A (NorA) efflux pump inhibitor 5'-

methoxyhydnocarpin (Stermitz *et al*., 2000). Screening for efflux pump inhibitors in plant extracts using bioassays designed to detect synergy with conventional drugs led to the isolation of *N-trans*-feruloyl 4'-*O*-methyldopamine from the methanolic extract of *Mirabilis jalapa* L**.** This molecule blocked NorA and thus significantly improved the activity of norfloxacin against *S. aureus (*Stermitz *et al*., 2000).

Other plant-derived potentiators include those that enhance antibiotics targeting cell wall synthesis and bacterial membranes. A major fraction of essential oils from plant extracts is composed of terpenoids which are a significant component in plant essential oils with antimicrobial effect. Synergy between major classes of clinically relevant antibiotics and sesquiterpenoids such as farnesol, nerolidol and others has been demonstrated (Gonzalez-Lamothe *et al*., 2009).

1.5.3.2 Plant-derived products as inhibitors of virulence in bacteria

Plant products such as those obtained from cranberry extracts and garlic extracts can be utilized as virulence attenuators. Virulence attenuation was demonstrated by first screening for quorum sensing inhibitory plant extracts. This was achieved by the design of reporter genes fused to quorum sensing-controlled promoters. Quorum sensing systems synchronize the infection process through the production of small diffusible signalling molecules that accumulate with increasing bacterial cell density (Novick and Geisinger, 2008). Garlic extract has been shown to be a potent quorum sensing inhibitor. The extract from this plant has been shown to reduce *Pseudomonas aeruginosa* biofilm resistance to tobramycin treatment (Rasmussen *et al*., 2005).

Extracts from cranberry fruit (*Vaccinium macrocarpon*) inhibited *E. coli* by down-regulation of its adhesion genes (Pages *et al.*, 2008) suggesting membrane disturbance as the mode of action. The effects could be due to constituents of cranberry extract such as condensed tannins (flavonoids) and phenolics that possibly act as iron chelators.

Tomatidine (from tomato), the aglycon version of the phytoanticipin tomatine have demonstrated bioactivity against *S. Aureus* (Bouarab *et al*., 2007). Although the MIC of the compound was high (>128 µg/mL), tomatidine inhibited hemolysin production by *S. aureus* on blood agar plates. Transcriptional analyses of *S. aureus* exposed to tomatidine showed down-regulation of many extracellular toxins, including alpha-hemolysin and deltahemolysin, serine proteases, lipases and nucleases. This modulation of gene expression was obtained using tomatidine concentrations as low as 1.28 µg/mL and suggests a possible application of tomatidine as a virulence attenuator.

1.6 Traditional methods of preparing herbal remedies

In traditional medicine, herbal remedies are prepared in several ways which usually vary based on the plant utilized, and the infection being treated. According to Taylor (2004), these methods include: infusions (hot preparations), decoctions (boiled preparations), and macerations (cold-soaking). These may be prepared using water or alcohol as the elution solvent (Taylor, 2004). Infusions are typically used for delicate herbs, leaves and tender plants. Water is boiled and poured over the herb(s), then

covered and allowed to steep (elute) for 10-15 min. Decoctions are usually the method of choice when working with tougher and more fibrous plants, barks and roots. Instead of just steeping it in hot water, the plant material is boiled for a longer period to soften the harder woody material and release its active constituents.

Some active chemicals found in plants are not soluble in water; therefore, a hot water-based preparation may not extract these chemicals (Taylor, 2004). Such chemicals may however be readily soluble in alcohol. Most plant components active against microorganisms are aromatic or saturated organic compounds and are therefore obtained through initial ethanol or methanol extraction. This is why the best method of preparing plant extracts has been the tincture (Taylor, 2004). A tincture is a preparation obtained using alcohol. This method is used when plants have active chemicals that are not soluble in water, and/or when a larger quantity is prepared for convenience and for longer term storage. With the tincture method, the dried herb is put in a glass jar with a tight-fitting lid; alcohol is added and left for 1 week while shaking occasionally (Taylor, 2004). The preparation is then strained through a cloth-sieve for clarification before use. In preparing macerations, the fresh or dried plant material is covered in cool water and soaked overnight. The herb is strained and the liquid collected. Normally this is used for very tender plants with heat and alcohol sensitive chemicals. Other methods include preparing plants in hot baths (in which the patient is soaked or bathed, inhalation of powdered plants (like snuff), steam inhalation of various aromatic plants boiled in hot water, and even aromatherapy).

1.7 Optimization of Methods

1.7.1 Choice of solvents

Successful determination of biologically active compound from plant material is largely dependent on the type of solvent used in the extraction procedure. According to Eloff (1998), properties of a good solvent in plant extractions and elution include low or no toxicity, ease of evaporation at low temperature, promotion of rapid physiologic absorption of the extract, optimal pH range, preservative properties of the solvent and ability to maintain the active compound in a stable form (eliciting no product dissociation). As the end product in extraction will contain traces of residual solvent, the solvent should be non-toxic and should not interfere with the bioassay (Ncube *et al*., 2008).

1.7.2 *In vitro* antimicrobial susceptibility testing

Although current standard antimicrobial susceptibility testing methods approved by various organizations like Clinical Laboratory Science (CLSI) for guidelines of antimicrobial susceptibility testing of conventional antibiotics exist, these are not applicable to plant extracts and modifications have to be made (Miyasaki *et al*., 2010). Standard antibiotic susceptibility tests are classified into diffusion and dilution methods. Diffusion tests include agar well diffusion and agar disk diffusion, while dilution methods include agar dilution, broth microdilution and broth macrodilution techniques. Determination of antimicrobial activity by agar diffusion necessitates a certain degree of hydrophilicity of the experimental substances which may not be achieved in such crude extracts with diverse chemical compounds. Attempts to

simulate the Kirby-Bauer disc diffusion methodology by creating paper discs impregnated with herbal extracts is also not always the best approach for studying the antimicrobial properties of an extract as both the absorption and diffusion coefficients of the paper discs are usually not pre-defined (Miyasaki *et al*., 2010).

1.7.3 Extract sterilization and Toxicity testing

Plant extract sterilization is an important practice so as to avoid microbial contamination that would complicate results interpretation. Sterilization can be achieved by physical, chemical and physiochemical means. The sterilization procedure should however not pose the risk of degrading the active compounds. Sterilization by autoclave poses the risk of heat degradation while sterilization by UV rays may introduce chemical transformations of the active components (Al-Bakri and Afifi, 2007). According to regulatory guidelines (ASTM, 2005), the filtrate from a 0.2-µm-pore-size sterilizing-grade membrane should filter all bacteria and ensure sterility. Membrane filters are however expensive, have little loading capacity and are fragile, a challenge which makes investigators to use non-sterile extracts.

1.8 Statement of the Problem

Since their discovery, antimicrobial agents have substantially reduced the threat posed by infectious diseases. However, the escalation of multidrug resistance in bacteria in recent years has seriously jeopardized these gains. Increased usage of antimicrobial agents to treat bacterial infections has led to the emergence of multi drug resistant (MDR) strains (Rodriguez *et al*., 2006). Gram negative isolates cause

more infections but are less susceptible to herbal extracts than Gram positive bacteria. Such strains are resistant to first line of treatments and also the more expensive second and third-line antibiotics. The high cost of such replacement drugs and the toxicological effects are prohibitive and are out of reach for many Kenyans. Furthermore, newer antimicrobials are losing their effectivity fast due to transfer of resistance markers from resistant to susceptible bacterial strains. This has necessitated a search for new antimicrobial substances from other sources especially plants, which produce diverse chemical compounds with different biological activities (Lewis and Ausubel, 2006). However, efficacy of the selected plant extracts is not laboratory tested against clinical isolates and their MICs are not determined. Additionally, it is not known if the use of such extracts in sub-lethal concentrations would stimulate or inhibit conjugal transfer of resistance determinants in the bacteria.

1.9 Justification

Many plants have been found to cure urinary tract infections, gastrointestinal disorders, respiratory diseases and cutaneous infections, caused by bacteria often known to resist various classes of conventional antibiotics (Somchit *et al.,* 2003). It is expected that plant extracts exhibiting effective inhibition to microbial growth may provide cheaper and more affordable alternatives for infection management. Moreover, the more efficient inhibition and lysis of bacteria resulting from the synergistic reactions of the active ingredients in plant extracts decreases the risk of progressively increasing antibiotic resistance. Thus, there is a need to evaluate medicinal plants for their antimicrobial potential against bacteria known to resist a

wide spectrum of conventional antibiotics either as antibiotics, detergents or antiseptics for infection control. McMahon *et al*. (2007) showed that the use of the tea tree oil in sub-optimal concentrations elicits basal resistance to conventional antibiotics. It is important to determine whether this phenomenon is observed in the selected herbal products. This study aimed at investigating antimicrobial properties of plant extracts against MDR clinical strains.

1.10 Null Hypothesis

Herbal extracts from the 10 plants have no antimicrobial effect on the MDR Gram-negative bacterial strains.

1.11 Objectives

1.11.1 General Objective

To evaluate herbal extracts from ten commonly used medicinal plants for potential antimicrobial effect on selected Gram-negative bacteria with known multi-drug resistance phenotypes.

1.11.2 Specific Objectives

1. To determine the susceptibitility profiles of test strains to conventional antibiotics and characterize the molecular nature of their resistance.
2. To determine the MICs of the herbal extracts on Gram-negative MDR bacteria.

3. To investigate effect of herbal extracts on conjugative transfer of antibiotic resistance determinants at sub-lethal concentrations.
4. To investigate if baseline resistance to conventional antibiotics is elicited by sub-lethal amounts of herbal extracts.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Source of plants tested

Plants were collected from Juja, located in lowland areas in the Eastern parts of Thika West District, Central Province, Kenya. Juja lies at latitudes 1° 10' 60S and Longitude 37° 7' 0E with an altitude of 1,060 meters above sea level. The area is generally semi arid and receives low rainfall of 856mm with a bimodal distribution. The primary peak of rainy season is in April and a secondary one in November. There is a dry period of about 4 months from June to October and a relatively shorter one extending from December to February (Muchena *et al*., 1978). The mean annual temperature is 20°C with the mean maximum temperature being 30°C. Relative humidity ranges from 57% in February to 74% in July. Evaporation rate ranges from 2.6mm in July to 6.3mm in February.

2.2 Test strains

A total of 27 test strains were used in various tests. These isolates included; *E. coli* (seven), *Klebsiella pneumoniae* (seven), *Salmonella typhi* (seven) and six strains of *Pseudomonas aeruginosa*. The bacterial isolates were identified by conventional techniques (Bergey, 1930). Susceptibility of the test strains to conventional antibiotics was determined by the disc diffusion technique and interpreted using the Clinical and Laboratory Standards Institute guidelines (CSLI, 2008).

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2.3 Medicinal plants

2.3.1 Collection and preparation of plant materials for extraction

The extracts tested in these experiments were obtained from plants traditionally used for the treatment of bacterial infections by Kenyan communities (Table 1). The 10 plants were selected on the basis of existing information on their use as remedies for bacterial infections (Table 1). The leaf, stem-bark and root samples were harvested, packed in clean sterile manila papers, labelled with a voucher specimen and transported to the laboratory for further analysis. The plant materials were oven-dried at 25°C and weighed every day until a constant weight was attained, after which they were chopped and ground to fine powder using a grinder. The herbal extracts were obtained from the ground powder through organic (methanol) and inorganic (water) solvents extraction.

2.3.2 Water extraction

Warm water extraction was done to simulate the traditional decoction method of preparing herbal preparations following a method modified from Okeke *et al.* (2001). Sixty (60) g of the ground powder was soaked in 300 ml sterile distilled deionised water at 50°C for 1 h and placed in an orbital shaker at 100 rpm for 24 h at 25°C. The resulting elute was membrane-filtered and lyophilized into granules using a lyophilizing machine (LyoBeta range, Telstar, UK). The extracts were stored at -20ºC until the time of use.

Table 1. Plant species screened for their antimicrobial activity

Plant name	Plant family	Phytochemicals	Medicinal uses
Clausena anisata (Wild) Benth.	Rutaceae	Flavonoids, tannins, saponins [f]	Stomach aches, whooping cough, malaria [f]
Aloe secundiflora L.	Aloceae	Phenolic compounds: chromone, anthraquinone or anthrone derivatives [k]	Disinfectant, treatment of Pneumonia, malaria, chest pains [k]
Strychnos henningsii Gilg.	Loganiaceae	Alkaloids, saponins [j]	Mouth antiseptic, wounds, gastrointestinal disorders, malaria [j]
Ajuga remota Benth.	Lamiaceae	Flavonoids, tannins and sterols [i]	Bacterial infections [i]
Ricinus communis L.	Euphorbiaceae	Alkaloids, saponins, tannins, phenolic [h]	Stomach aches, diarrhoea**,** boils, burns, worms, venereal diseases [h]
Zanthoxylum chalybeum Engl.	Rutaceae	Alkaloids and saponins [g]	Bacterial infections, malaria, colds, cough, toothache [g]
Tamarindus indica L.	Caesalpinaceae	Alkaloids, flavonoids, saponins, tannins [f]	Treatment of scabies, boils, diarrhoea, dysentery [f]
Warburgia ugandensis Sprague.	Canellaceae	Sequiterpene dialdehydes: warburganal, muzigadial, polygodial [e]	Constipation, treatment of bacterial infections, stomach aches, coughs, muscle pains, weak joints, body pains [e]
Terminalia brownii Tul.	Combretaceae	Tannins, saponins, flavonoids, alkaloids [c]	Diarrhoea, stomach ache, fevers, hepatitis, colds [d]
Azadirachta indica A. Juss	Maliaceae	Tannins, saponins, flavonoids, terpenoids, alkaloids [a]	Abdominal colic, treatment of wounds, boils, scabies, malaria, rheumatism [b]

[a]Krishnaiah *et al.* 2008; [b]Thakurta *et al.* 2009; [c]Osuga *et al*. 2006 ; [d]Zakaria *et al*., 2007; [e]Haraguchi, 1998; [f]Daniyan and Muhammad, 2008; [g]Olila *et al.* 2001; [h]Raji *et al.*, 2006; [i]Bekele, 2008; [j]Michel *et al*. 1999; [k]Mascolo *et al.*, 2004.

2.3.3 Organic extraction

Organic extraction of plant extracts was done by a method modified from Okeke *et al.* (2001). Sixty (60) g of the ground powder was soaked for 72 h in a beaker containing 300 ml of methanol (Analytical Grade, 99.9%, Sigma-Aldrich, UK) and placed in an orbital shaker (Basic Model, Cole-Parmer, UK) at 100 rpm at 25°C. The resulting extracts were membrane filtered on a whatman paper number 1 then the filtrate concentrated at 50ºC using a vacuum rotary evaporator (Basic Model, Buchi, UK). Excess methanol was evaporated by air-drying the paste at 25°C until a constant weight was attained. The extracts were stored at -20ºC until the time of use.

2.4 Phenotypic and molecular Characteristics of the clinical strains

2.4.1 Susceptibility profiles of test strains to conventional antibiotics

The susceptibility profiles of isolates to conventional antibiotics were determined by the disc diffusion technique using discs of known potency following manufacturers' instructions (Cypress diagnostics, Langdorp, Belgium). A single pure bacterial colony from a MacConkey plate (Oxoid) was picked and suspended in normal saline (0.85% NaCl) to a 0.5 McFarland standard (approximately 10^6 CFU/ml). The suspension was inoculated uniformly on Mueller-Hinton agar (Oxoid). The antibiotic disks were then placed equidistance from each other on the inoculated plate. The panel of antibiotics used included cefuroxime (30 µg), cefixime (5 µg), cefoxitin (30 µg), ceftriaxone (30

µg) and amoxicillin/ clavulanic acid (20/10 µg). Other antibiotics included in this panel were aztreonam (30 µg), norfloxacin (10 µg), nalidixic acid (30 µg), streptomycin (10 µg), neomycin (10 µg), minocycline (30 µg), chloramphenicol (30 µg), sulphamethoxazole (50 µg) and trimethoprim (5 µg). *E. coli* ATCC 25922 was used as a control for bacterial growth and potency of antibiotic discs. Susceptibility tests were interpreted using the Clinical and Laboratory Standards Institute guidelines (CSLI, 2008).

2.4.2 PCR amplification for resistance genes

Based on their susceptibility profiles, the test isolates were screened for the presence of β-lactamases (*bla-*$_{SHV}$ and *bla-*$_{TEM}$ genes) and mobile genetic elements (conjugative plasmids and integrons) using polymerase chain reaction (PCR) strategies following a method modified from Eftekhar *et al.* (2005). DNA used as template was prepared from overnight MH broth cultures incubated at 37°C with shaking. Cells were harvested by centrifugation and re-suspended in 1 ml 10 mM Tris/HCl (pH8·0) containing 1 mM EDTA. The cells were boiled for 10 min and the lysates separated by centrifugation at 12,000 × g for 3 min and stored at -20°C until further analysis. PCR was carried out in 50 µl reaction volumes containing 5 µl 10× concentrated PCR buffer [100 mM Tris/HCl (pH8·3), 500 mM KCl, 15 mM $MgCl_2$], 5 µl (10 pmol μl^{-1}) each of primer, 4 µl dNTP mix (2·5 mM each dNTP), 0.25 µl (5 U μl^{-1}) *Taq* DNA polymerase, 5 µl of template

DNA and 25.75 µl sterilized distilled water. The band sizes of the PCR products were identified using a molecular marker (Hyper ladder, Eurogentec). All PCR assays were performed using an automated thermal cycler (GeneAmp PCR System 9700; Applied Biosystems). PCR products were analysed by electrophoresis in 1.5% agarose gels, stained with ethidium bromide, visualized under UV light and recorded with the aid of a gel documentation system (Bio-Rad Laboratories, Hercules, Ca, USA). Detection of *bla-$_{SHV}$* and *bla-$_{TEM}$* genes was done using the forward primer OT3 (5'-ATG AGT ATT CAA CAT TTC CG-3') and the reverse primer OT4 (5'-CCA ATG CTT AAT CAG TGA GG-3') for TEM while the primers for SHV were OS5 (5'-TTA TCT CCC TGT TAG CCA CC-3') and OS6 (5'-GAT TTG CTG ATT TCG CTC GG-3') as forward and reverse primers respectively. Detection of class 1 integron was done using the forward primer Int1F (5'-GTTCGGTCAAGGTTCTG-3') and the reverse primer Int1R (5'-GCCAACTTTCAGCACATG-3') specific for the integrase gene, *intI*1, while a combination of qacEΔ1-F (5'-ATCGCAATAGTTGGCGAAGT-3') and sul1-B (5'-GCAAGGCGGAAACCCGCGCC-3') were utilised for the detection of the 3'-CS. The variable cassette region was analysed using a combination of In-F (5'-GGCATACAAGCAGCAAGC-3') and In-B (5'-AAGCAGACTTGACCTGAT-3').

2.4.3 Conjugation experiments

Mating experiments were done according to the protocol previously described by Finlay and Falkow (1988)**.** The clinical strains were used as donors while the *E. coli* strain J53

(resistant to sodium azide) was used as the recipient. The donors and the recipient were sub-cultured on MacConkey plates and incubated at 37°C overnight to obtain pure colonies. A single colony of the donor and the recipient were resuspended separately in normal saline to a McFarland standard of 0.5 and an inoculum of the ratio 1: 3 for donor and recipient respectively transferred to tubes containing 5ml MH broth. The tubes were then incubated at 37°C for 8 h without shaking. Transconjugants from each set were then selected on MacConkey plates supplemented with 100µl/ml ampicilin and 100µl/ml Sodium azide. Ampicillin was used so as to inhibit the growth of the recipient while Sodium azide was used to suppress the growth of donors. The plates were incubated at 37°C and checked for transconjugants after 8 h. Therefore, only transconjugants with combined resistance to ampicillin and sodium azide were recovered from these plates as they had acquired ampicillin resistance genes. Conjugation frequencies were determined by dividing the number of transconjugants per plate by the total number of recipients. The susceptibility of the transconjugants was determined using the same panel of antibiotic disks used for the donor strains.

2.4.4 Plasmid extraction and profiling

The method of extraction was by alkaline lysis (Birnboim and Doly, 1979) following the protocol recommended by the manufacture (Qiagen, Hilden, Germany). Discrete colonies of the test strains and transconjugants were obtained by streaking onto Muller

Hinton plates and incubated at 37°C for 8 h. A pea-sized amount of inoculum was scrapped from the plates and transferred to 1.5 ml Eppendorf tube containing sterile de-ionized water. All manipulations were carried out at room temperature. The Cultures were centrifuged for 10 min at 14,000 rpm in a table-top microcentrifuge and the supernatant discarded. The pelleted bacterial cells were resuspended in 250 µl buffer P1. 250 µl and 350 µl of buffer P2 and N3 were added respectively and mixed thoroughly by inverting the tube 4-6 times. The mixture was centrifuged for 10 min at 14,000 rpm and the supernatant applied to the QIAprep spin column by decanting. This was centrifuged for 2 min and the flow-through discarded. The QIAprep spin column was washed by adding 0.5 ml buffer PB, centrifuged for two min and the flow-through discarded. The QIAprep spin column was again washed by adding 0.6 ml buffer PE and centrifuged for two min. The flow-through was discarded and centrifuged for another 2 min to remove residual wash buffer. The DNA was eluted by placing the QIAprep column in a clean 1.5ml microcentrifuge tube. A volume of 50 µl EB buffer was added to the centre of each QIAprep spin column, left to stand for one min and centrifuged for 2 min. The plasmids profiles were subjected to electrophoresis on 1% agarose gel for 1 hour at 100 V. The plasmids of known sizes from *E. coli* V517 and *E. coli* R39 were run alongside as controls.

2.5 Antimicrobial Assays

2.5.1 Pre-Screening of the herbal extracts for antimicrobial activity against test strain

Pre-Screening of the herbal extracts for antimicrobial activity against test strain was done by a method modified from Okeke *et al.* (2001). The *E. coli* J53 strain was also used for initial screening of inhibition. A confluent lawn of the bacteria was prepared from a 0.5 MacFarland standard (approximately 10^6 CFU/ml) and placed in an incubator to dry. For each extract, a stock solution of 100 µg/ml was prepared. This was serially diluted to obtain various ranges of concentrations between 5 µg/ml to 100 µg/ml. 5 µl of the different extract concentrations was point inoculated using a micropipette (Eppendorf, Hamburg, Germany). Extract-free sterile distilled water and TE buffer (the diluents used for making the stock solutions of the plant extracts), were inoculated in another set of plates and used as negative controls. The plates were allowed to stand for at least 1 h at room temperature for the extracts to diffuse into the medium before incubation at 37°C for 12 h. Observation of a clear zone on bacterial lawn at the point of inoculation of the extract was interpreted as evidence of inhibition of bacterial growth. The diameter of these zones were measured and recorded to the nearest size in mm. The plants whose extracts exhibited an inhibition zone > 24 mm were selected for further testing of clinical isolates.

2.5.2 Determination of MICs and the sub-lethal concentrations of the herbal extracts

Determination of MICs was done by a method modified from Greenwood and Eley (1982). Duplicate tubes containing 2 ml MH broth were prepared. To one set of these tubes, 0.5 ml of the extracts in different concentrations of 5 µg/ml to 100 µg/ml were added to separate tubes before a uniform inoculum of 10^6 CFU/ml of the test isolates was added. Negative controls were set in a similar way but using extract-free distilled water or TE buffer. The preparations were incubated for 12 h at 37ºC with continous shaking at 100 rpm in an orbital shaker. The tubes were removed from the shakers and arranged sequentially according to the order of increasing concentration of the herbal extract. The tubes were assessed for evidence of bacteria growth by physical examination before streaking 1 µl of the preparations on MacConkey plates. The plates were then incubated for 12 h and the colonies counts determined. The tube containing the lowest concentration at which no colonies were observed was identified and defined as the Minimum Inhibitory Concentration (MIC). The tube containing the preparation at which the last visible colonies were obtained was defined as the highest amount of extract that does not inhibit bacterial growth. This concentration was therefore designated as the highest Sub-Lethal Concentration (SLC) of the extract. The SLC was used as the reference concentration in the habituation experiments.

2.6 Habituation to sub-lethal concentrations

This was done by a method modified from McMahon *et al*. (2007). Two sets of duplicate tubes containing 2 ml MH broth were prepared. The sub-lethal concentration of an extract was added and inoculated with approximately 10^6 CFU/ml bacterial cells of the clinical isolates or controls ATCC *E. coli* strains 25922 and J53. Negative controls were prepared using similar sets of bacterial preparations but instead of the extracts, deionised sterile distilled water or sterile TE buffer (pH 8) were used as controls. All preparations were incubated for 72 h at 37°C with constant shaking at 100 rpm after which colony counts were determined on MacConkey agar.

2.7 Effect of pH on antimicrobial potential of the extracts

Two sets of extract solutions were prepared by dissolving the extract in TE buffer of pH 3, 8 and 11. Different titres of extracts were separately added to duplicate tubes containing 2 ml MH broth and inoculated with 10^6 CFU/ml of the test isolates. The extract suspended in TE buffer of pH 8 (the pH of the buffer used as diluent in standard experiments) was used as the positive control. All preparations were incubated for 8 h at 37ºC, after which colony counts were determined on MacConkey agar.

2.8 Determination of the effect of extracts on bacteria

The herbal extracts were investigated for their effect on the test strains. Two effects were investigated; inhibition of growth (ability to hinder establishment of colonies) and lysis

(clearance of preformed bacterial colonies). A confluent lawn of the tester bacteria was prepared from a 0.5 MacFarland standard and placed in an incubator for 5-10 min at 37ºC to dry. Ten (10) µl of the MIC equivalent of a herbal extract was swabbed over the plate after the 10 minutes to cover the bacterial inoculum previously applied. The plates were then incubated for 8h at 37ºC. Absence of bacterial growth on the plates upon incubation was interpreted as evidence of inhibition of bacterial growth by the extracts. To test for lysis of bacterial cells, plates with an established confluent lawn of bacteria were spotted with 10 µl of the MIC equivalent of a herbal extract and incubated for 8h at 37ºC. Evidence of zones of clearance on the existing bacterial lawn was then assessed. Plaques and zones of discontinuous growth was taken as evidence of lysis of preformed colonies

2.9 Interactive effect of different plant extracts

Sets of duplicate tubes containing 2 ml MH broth were prepared. To each tube, equal titres of two different plant extract preparations previously identified to exhibit inhibitory effect on *E. coli* J53 were added. From each extract, the half-MIC equivalent concentration was added. An inoculum of approximately 10^6 CFU/ml of test bacteria was then added to this preparation. For each of these experiments, two positive controls were prepared but to each tube, individual test extracts were added separately. All preparations were incubated for 12 h at 37°C, after which colony counts were determined.

2.10 Screening for effect of the herbal extracts on conjugation

In order to investigate whether the plant extracts had an effect on conjugative efficiency, *in vitro* mating experiments in which the herbal extracts were added to the tubes containing the clinical strains (donor) and recipient (*E. coli* J53) mixed for mating were designed while another set of similar sets were designed in a similar manner but without addition of the extracts. Mating experiments were done according to the protocol previously described by Finlay and Falkow (1988)**.** Three sets of tubes containing 5ml MH broth and inoculated with a donor and recipient were prepared. To the first set, a sublethal amount of the extract was added. To the second set, the MIC equivalent of the herbal extract was added, and to the third set, no extract was added. Therefore, no bacterial growth was expected in the sets of tubes to which the MIC-equivalent of the extract was added while limited growth was expected from the tubes with the sub-lethal concentration of the extracts. The third set without any extract served as the positive control and normal conjugation frequencies were expected. All sets of tubes were then incubated at 37°C for 12 h without shaking.

2.12. Ethical Consideration

This being a trial project, no information concerning the patients from which the test isolates had been obtained was made available to us and neither did our collaborators disclose the health institution from which the strains were obtained. Based on the ethical considerations governing the execution of this ongoing project, all the microbial assays

were done at the KEMRI- CMR laboratory. Permission to carry out the study was obtained from Kenya Medical Research Institute (KEMRI) Scientific Committee and Ethical Review Committee (Kiiru *et al*. KEMRI SSC No. 1177, Appendix 7).

2.13 Data Analysis

Data entry management and preliminary summaries were done in Microsoft Excel Spreadsheet. Antibiotic susceptibility tests were interpreted according to the guidelines provided by the manufacture's interpretation charts (Cypress diagnostics, Langdorp, Belgium). Bioline Hyperladder 1 was used as the standard marker for PCR products (*bla*-$_{TEM,}$ *bla*-$_{SHV}$ and class 1 integron genes) while plasmids of known sizes from *E. coli* V517 and *E. coli* R39 were used as size markers for plasmid profiling. Data from repeated experiments from antimicrobial assays were subjected to paired T test using SPSS version 17.0 Statistical software. Mean colony counts were obtained by averaging the colony counts for the duplicate sets and their standard error (SE) obtained. Conjugation frequencies were determined by dividing the number of transconjugants per plate by the total number of recipients. Data from antibiotic susceptibility profiles of the exposed and non-exposed transconjugants was subjected to paired T test. Probability value of $P<0.05$ was used for entire tests to show statistical significance of mean values for parameters analyzed.

CHAPTER THREE

RESULTS

3.1 Phenotypic and Molecular Characteristics of the Clinical Strains

3.1.1 Susceptibility profiles of test strains to conventional antibiotics

All the 27 test isolates had combined resistance to at least; two quinolones, two or more cephalosporins, two aminoglycosides, quinolones, tetracyclines, trimethoprim, and to at least one or more β- lactam/β- lactamase inhibitor. All 27 isolates were resistant to ampicillin and 26 isolates resistant to augmentin while only 8 isolates were resistant to cefoxitin (Table 2).

3.1.2 PCR screening for resistance genes

All the 27 clinical isolates apart from E_7 (*E. coli* isolate 7), S_2 (*S. typhi* isolate 2), K_3 (*K. pneumoniae* isolate 3), K_5 (*K. pneumoniae* isolate 5) and P_4 (*P. aeruginosa* isolate 4) carried the *bla-*$_{TEM}$ gene of approximately 1000bp (Plate 1). Similarly, all isolates positive for the *bla-*$_{TEM}$ gene carried the *bla-*$_{SHV}$ gene of approximately 1000bp apart from isolate K_1. The isolate K3 was also carried the *bla-*$_{SHV}$ gene (Plate 2).

The class 1 integrons were found in 20 out of 27 of the test isolates (Figure 6). The band sizes of the class 1 integrase gene (*intl*1) measured approximately 2000 kb while the

variable cassette region (VCR) ranged between 1600bp – 10,000bp. One isolate, K_2, was positive for *intI*1 but negative for VCR (Plate 3).

Table 2: Susceptibility of 27 clinical MDR Gram-negative strains to 15 conventional antibiotics

	Test Strains																									
Antibiotics	**E_1**	**E_2**	**E_3**	**E_4**	**E_5**	**E_6**	**E_7**	**K_1**	**K_2**	**K_3**	**K_4**	**K_5**	**K_6**	**K_7**	**S_1**	**S_2**	**S_3**	**S_4**	**S_5**	**S_6**	**S_7**	**P_1**	**P_2**	**P_3**	**P_4**	**P_5**	**P_6**
NOR	S	R	R	S	S	S	R	S	R	R	R	R	S	R	S	R	S	S	R	S	S	S	S	S	S	S	S
SMX	R	R	R	R	R	R	R	S	R	R	R	R	S	R	R	R	R	R	R	R	R	R	S	R	R	S	R
NA	R	R	R	R	R	S	R	S	R	R	R	R	R	R	R	S	S	S	R	S	S	R	R	S	S	R	R
TM	R	S	S	R	R	R	S	S	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R
C	R	R	R	R	R	S	R	S	S	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R
N	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	S	S	S	S	S	R	R	S	S	S	S
S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	S
MN	R	R	R	R	S	R	I	S	S	R	R	R	R	R	R	R	R	S	R	R	R	R	R	S	R	R	R
CXM	R	R	R	R	R	R	R	S	R	S	R	R	R	R	S	S	S	S	S	S	S	S	R	S	S	R	R
CFM	R	R	R	R	R	R	R	S	R	S	R	R	S	R	S	R	S	S	S	S	S	S	R	S	S	R	R
CRO	R	R	R	R	R	R	R	S	R	S	R	R	R	R	S	R	S	S	S	R	S	R	R	S	S	S	R
ATM	R	R	R	R	R	R	R	S	R	R	R	R	S	R	S	R	S	S	S	S	R	S	S	S	S	R	R
FOX	R	S	S	S	S	S	S	S	S	S	S	R	R	S	S	S	S	R	S	S	S	R	R	S	S	R	R
AUG	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
AMP	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R

R: Resistant (intermediate phenotypes were recorded as resistant); S: Sensitive.

Antibiotics:

NOR: Norfloxacin
SMX: Sulfamethoxazole
NA: Nalidixic acid
TM: Trimethoprim
C: Chloramphenical
N: Neomycin
S: Streptomycin
MN: Minocycline
CXM: Cefuroxime
CRO: Ceftriaxone
ATM: Aztreonam
FOX: Cefoxitin
AUG: Augmentin
AMP: Ampicilin

Gram-negative clinical isolates of the species: *E. coli:* E_1-E_7; *K. pneumoniae:* K_1-K_7; *P. aeruginosa:* P_1-P_6; *S. typhi:* S_1-S_7.

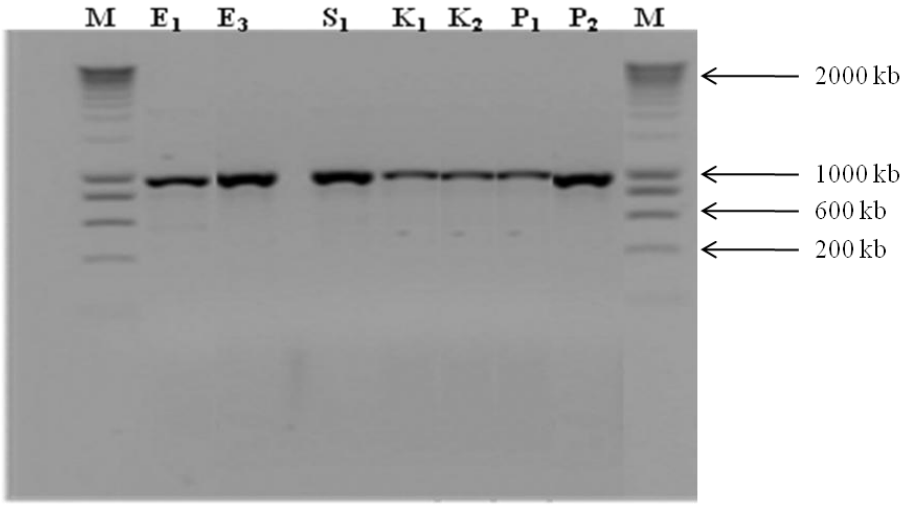

Plate 1: The TEM genes from clinical MDR Gram-negative isolates. M: Molecular marker (Hyper ladder); $E_1$and E_3: *E. coli* isolates; S_1: *S. typhi* isolate; K_1 and K_2: *K. pneumoniae* isolates; P_1 and P_2: *P. aeruginosa* isolates.

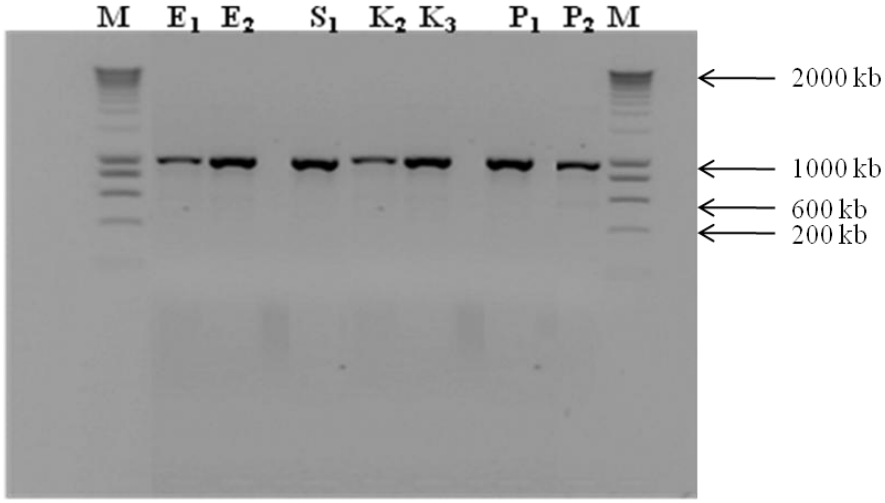

Plate 2: The SHV genes from clinical MDR Gram-negative isolates. M: Molecular marker (Hyper ladder); $E_1$and E_2: *E. coli* isolates; S_1: *S. typhi* isolate; K_2 and K_3: *K. pneumoniae* isolates; P_1 and P_2: *P. aeruginosa* isolates.

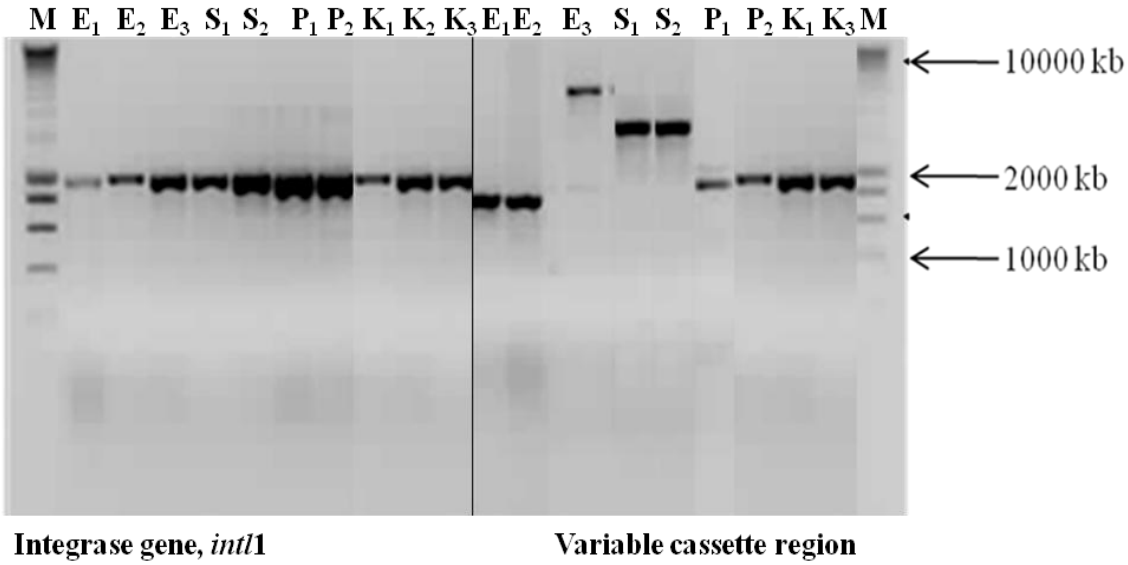

Plate 3: Class I integrons from clinical MDR Gram-negative isolates. M: Molecular marker (Hyper ladder); E_1, E_2 and E_3: *E. coli* isolates; K_1, K_2 and K_3: *K. pneumoniae* isolates, S_1 and S_2: *S. typhi* isolates; P_1 and P_2: *P. aeruginosa* isolates.

3.1.3 Plasmid profiling

The clinical isolates of *S. typhi* had plasmids that ranged between 3.5 to 53.7 kb. Only one plasmid of molecular weight 40.3 kb was transferred to the transconjugants in *S. typhi* isolates. One isolate, S_1 (*S. typhi* isolate 1), did not transfer the plasmid to the transconjugant (Plate 4). The clinical isolates of *E. coli, K. pneumoniae* and *P. aeruginosa* had plasmids that ranged between 1.4 kb to 15.9 kb and only two or three of such plasmids were transferred to the transconjugants. Two *P. aeruginosa* isolates, P_2 and P_3 did not transfer the plasmids to the transconjugants (Plate 5).

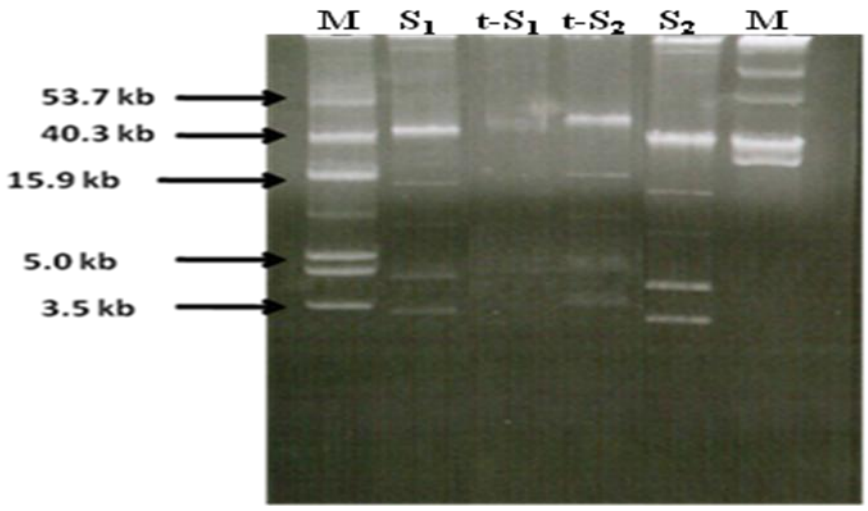

Plate 4: Plasmid DNA from MDR *Salmonella typhi* isolates. M_1 and M_2: plasmid from *E. coli* V517 and *E. coli* R39 respectively (used as plasmid size markers); t-S_2: transconjugant positive for *S. typhi* isolate S_2.

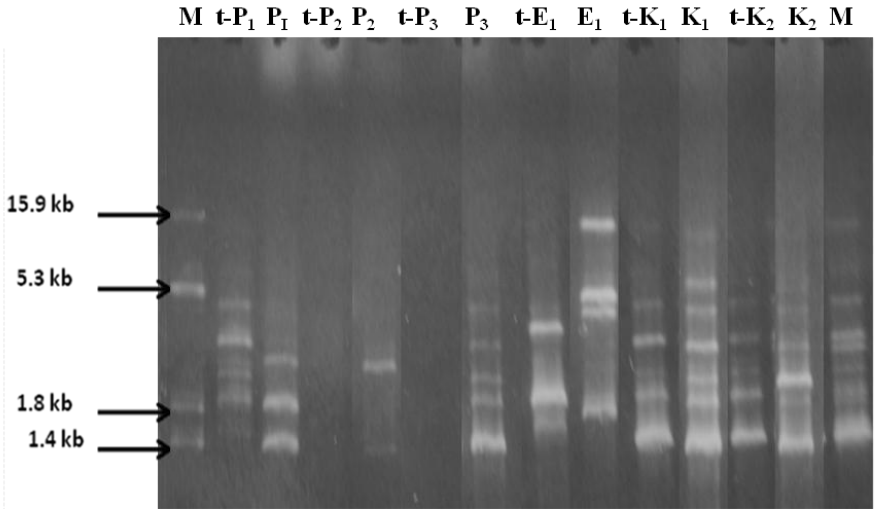

Plate 5: Plasmid DNA from MDR *E. coli, Klebsiella pneumoniae* and *P. aeruginosa* isolates. t-P_1, transconjugant for *P. aeruginosa* isolate P_1, t-E1: transconjugant for *E. coli* isolate E_1; t-$K_1$and t-K_2: transconjugants for *Klebsiella pneumoniae* isolates K_1 and K_2.

3.2 Antimicrobial Assays

3.2.1 Pre-Screening of the herbal extracts for antimicrobial activity

The bacterial strain, *E. coli* J53, was used in the preliminary screening of the herbal extracts for their potential to inhibit bacterial growth. The extracts from the 10 plants exhibited antibacterial effect and this depended on the plant species, part of the plant used, method of extraction and the concentration of extract used (Table 3). Methanol extracts of *W. ugandensis* had antibacterial activity even at concentrations as low as 5 µg/ml. There was a correlation between the concentration used and the zone of inhibition (t-test, $p<0.05$).

Methanol extracts from all the plants exhibited better activity than those extracted using water (t-test, $p<0.05$). The negative controls inoculated with distilled water or TBE pH 8 without extracts showed no inhibitory effect. Extracts from *W. ugandensis* showed a better activity compared with those from other plants (t-test, $p<0.05$) and the root extracts from this plant were more effective than those from the leaf preparations (t-test, $p<0.05$) but of equal effectivity with those from the stem-bark (t-test, $p>0.05$). The inhibition zone diameter at 50 µg/ml methanol extract of the root was 30 mm while that of the stem-bark and leaf was 28 mm and 16 mm respectively. The water preparation of this plant gave lower inhibition zones of 22 mm, 20 mm and 14 mm for the root, stem-bark and leaf extracts respectively at the same concentration (Table 3).

Extracts from the leaves of *A. secundiflora* had the least inhibitory activity with an inhibition zone of 10 mm at 50 µg/ml concentration for both methanol and water extracts followed by extracts from *R. communis* with an average inhibition zone of 11mm at same concentration (Table 3).

Table 3: Diameter of inhibition zones at 50 µg/ml of 10 plant extracts against *E. coli* J53.

Plant	Plant Part	Diameter of Inhibition Zones (mm)	
		Methanol Extracts	Water Extracts
W. ugandensis	Root	30*	22
	Stem-bark	28*	20
	Leaf	16	14
T. brownii	Root	16	17
	Stem-bark	26*	22
	Leaf	27*	23
A. indica	Root	17	16
	Stem-bark	25*	22
	Leaf	24*	21
C. anisata	Root	23	21
	Stem-bark	25*	20
	Leaf	15	14
S. henningsii	Root	24*	20
	Stem-bark	21	20
	Leaf	17	12
Z. chalybeum	Root	14	10
	Stem-bark	17	10
	Leaf	15	11
A. remota	Root	15	10
	Stem-bark	17	11
	Leaf	16	10
T. indica	Root	15	11
	Stem-bark	16	11
	Leaf	17	10
R. communis	Root	11	10
	Stem-bark	10	10
	Leaf	11	11
A. secundiflora	Leaf	10	10

*Inhibition zones ≥24 mm at 50 µg/ml of plant extract.

The plants whose extracts of 50 µg/ml produced of an inhibition zone of greater than 24 mm were selected for further testing using clinical strains and their MICs determined. The selection of this zone size as a basis of further testing was meant to provide a convenient cut-off point that would generate un-equivocal results in further experiments. The plants whose extracts met this criterion were: *W. ugandensis; T. brownii; A. indica; C. anisata* and *S. henningsii* (Table 3).

3.2.2 Characterization of plants with inhibitory effect on clinical strains

The five plants whose extracts produced an inhibition zone ≥24 mm at 50 µg/ml concentration were further tested using clinical Gram-negative isolates. Although extracts from these plants had been tested to give inhibitory effect on *E. coli* J53, only methanol extracts from the root and stem-bark of *W. ugandensis* were inhibitory to the MDR strains. The antibacterial activity on these clinical strains by the methanol extracts of *W. ugandensis* was significantly higher than that of the other plants such as *T. brownii* ($p<0.05$); *A. indica* ($p<0.05$); *C. anisata* ($p<0.05$) and *S. henningsii* ($p<0.05$) as shown in Figure 5.

The colony counts from non-effective extracts were not significantly different from those of the negative controls (extract-free diluents) and there were no colonies at 50 µg/ml of *W. ugandensis* methanol extract preparation. Mean colony counts were obtained by averaging the colony counts for the duplicate sets at 50 µg/ml of the extracts and their standard error (SE) obtained (Figure 5). Inhibition of the other clinical isolates was similar to that of *E. coli* isolate, E_1. The results on comparative

inhibitory characteristics of the different plants are shown in Figure 5 while statistical comparisons of this data is provided in Appendix 1.

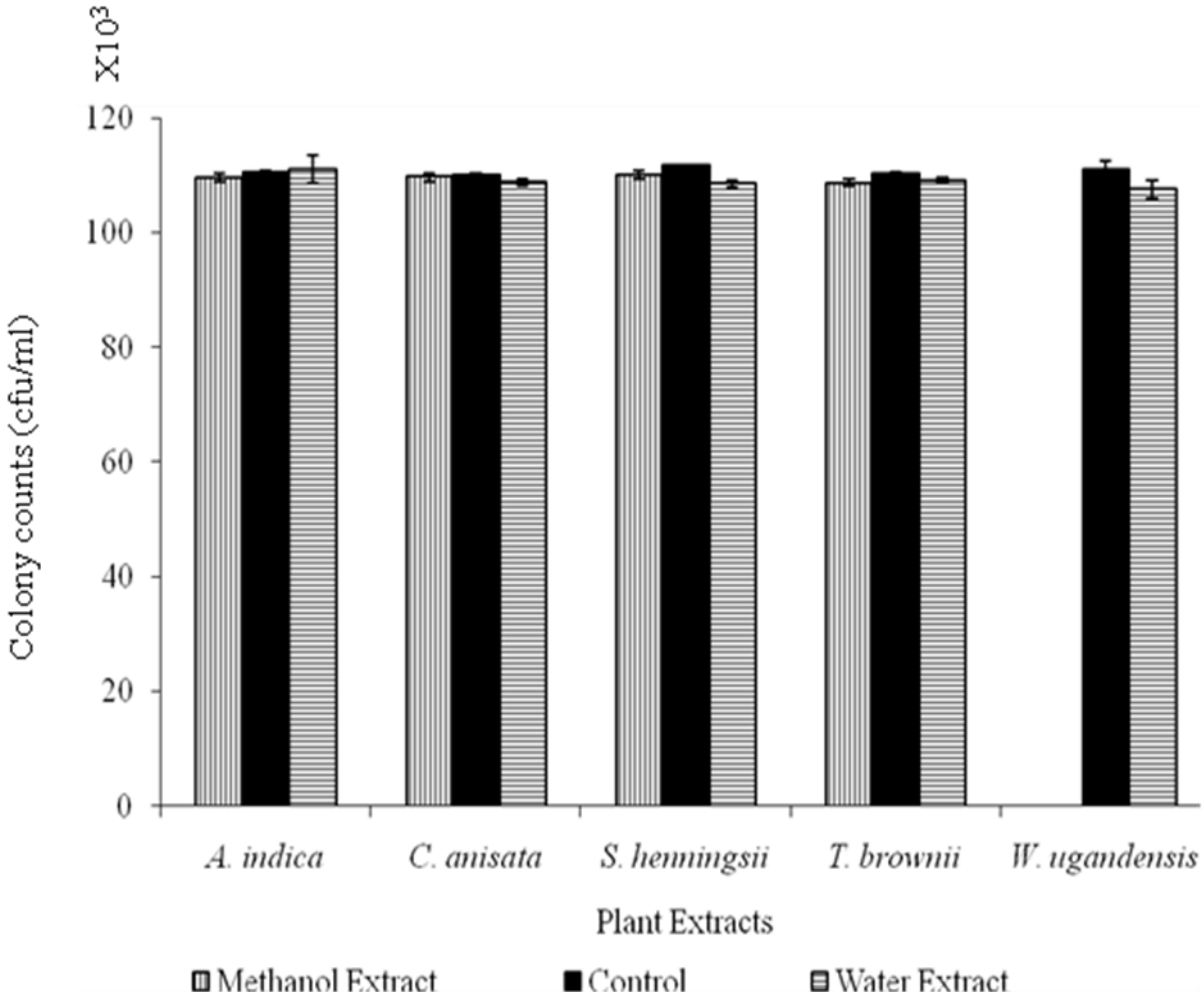

Figure 5: Inhibitory characteristics of the total extracts from five plants against a clinical *E. coli* isolate, E_1.

3.2.3 Comparison of inhibitory characteristics of different parts of *W.ugandensis*

Based on colony counts, methanolic extracts from the stem-bark of *W. ugandensis* exhibited better inhibitory properties than those obtained using water extraction (t-test, $p<0.05$) as shown in Figure 6. Similarly the root extracts obtained using methanol from this plant were more effective than those obtained using water extraction method (t-test, $p<0.05$), However, there were no significant differences in the methanolic and water extracts from the leaf (t-test, $p>0.05$). The inhibition characteristics of root and stem-bark extracts obtained using methanol were not significantly different (t-test, $p>0.05$) as shown in Appendix 2. The mean colony counts were obtained by averaging the colony counts for the duplicate sets at 50 µg/ml concentration of *W. ugandensis* extracts against a clinical *S. typhi* isolate, S_1, and their standard error (SE) obtained (Figure 6)**.** Inhibition was similar for the other clinical isolates. A detailed statistical comparison of the inhibitory characteristics of the different plant parts is shown in Appendix 2.

3.2.4 Inhibitory characteristics of *W. ugandensis*

There was a gradual decrease in the number of colonies as the amount of titre (root and stem-bark) methanol extracts of *W. ugandensis* increased (t-test, $p<0.05$) as shown in Figure 7. The colony counts from all other plants were not significantly different from those of the negative controls indicating that there was no inhibition. Colony counts of root and stem-bark extracts from *W. ugandensis* obtained using methanol decreased as extract titres increased**.**

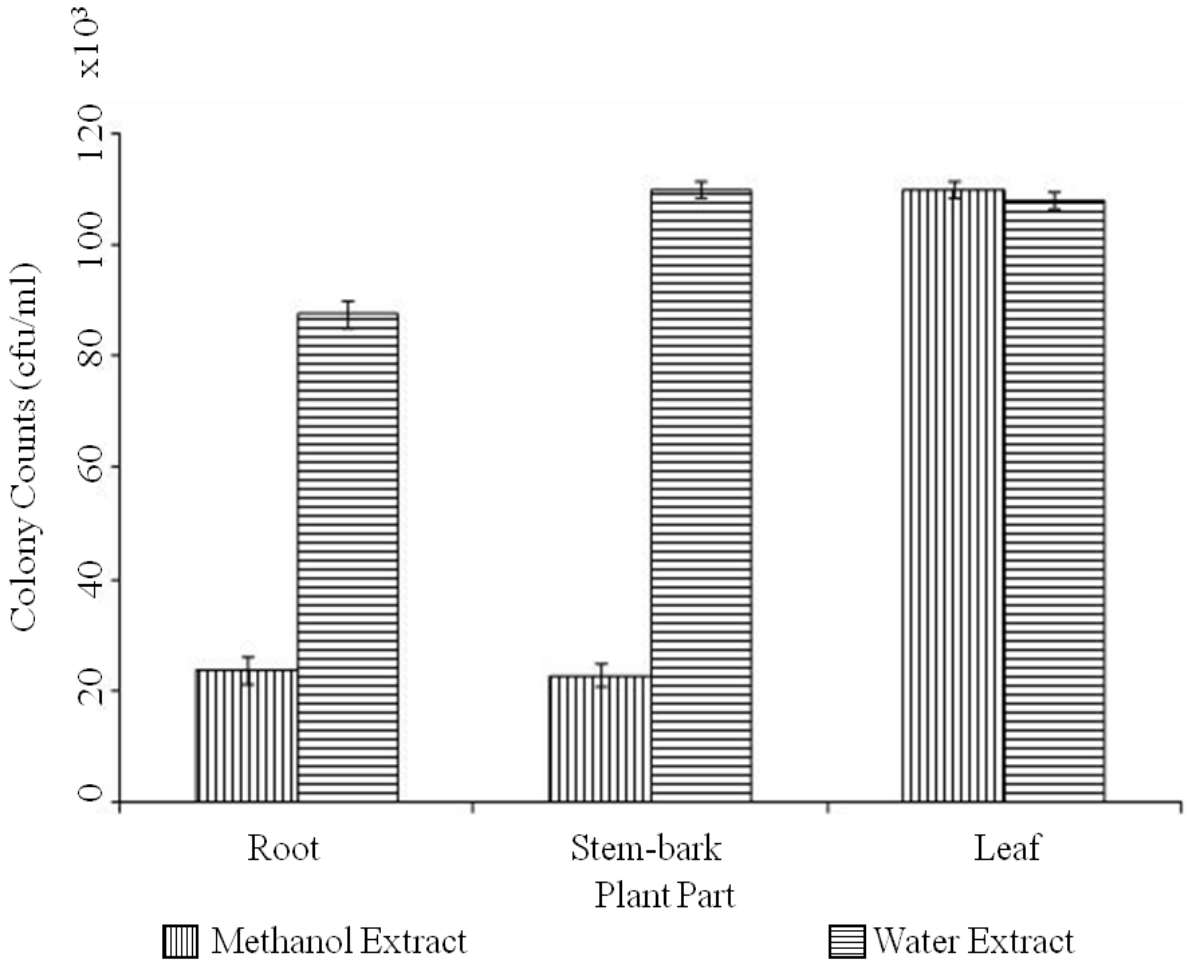

Figure 6: Differences in the effectiveness of water and methanol extracts of *W. ugandensis* against a clinical *S. typhi* isolate, S_1.

The mean colony counts were obtained by averaging the colony counts for the duplicate sets at 50 µg/ml concentration of *W. ugandensis* extracts against a clinical *K. pneumoniae* isolate, K_1, and their standard error (SE) obtained (Figure 7)**.** Inhibition was similar for the other clinical isolates. Details are provided in Appendices 3, 4 and 5.

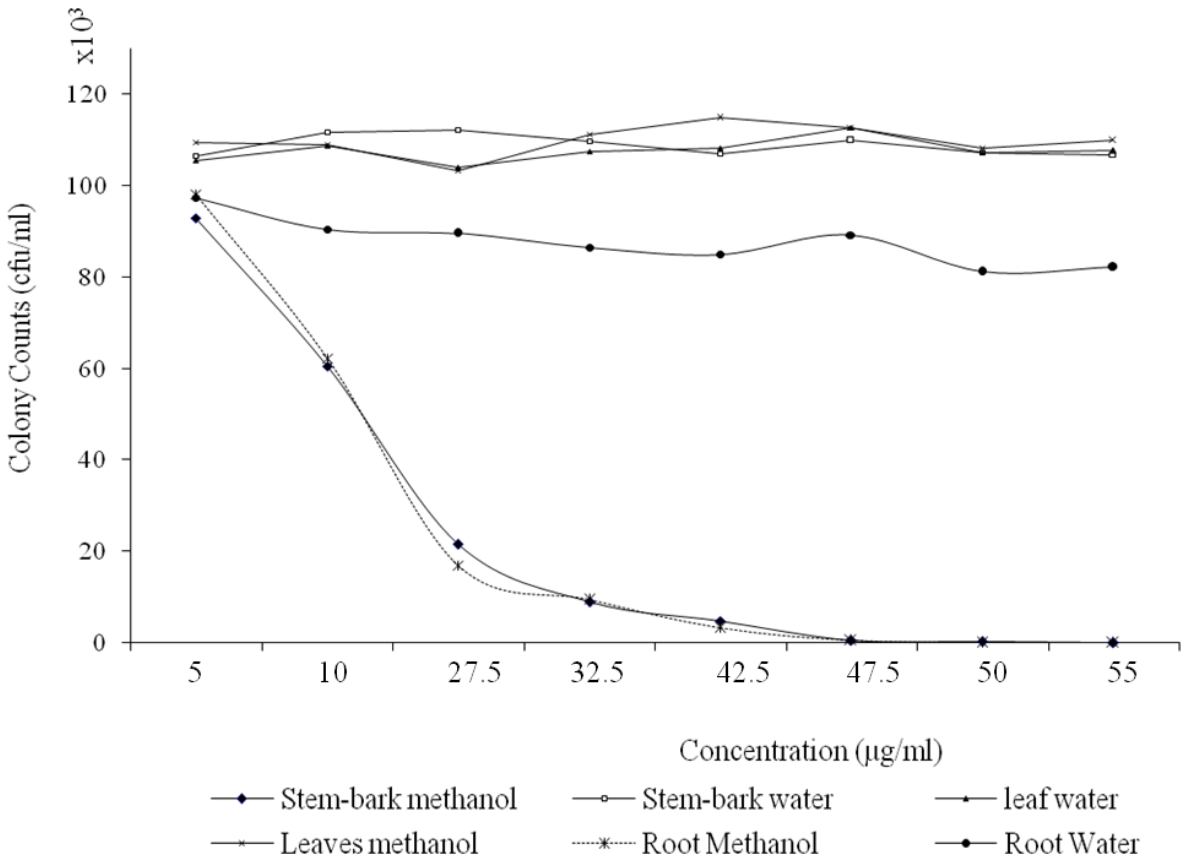

Figure 7: Growth characteristics of a clinical *K. pneumoniae* isolate, K1, subjected to different titres of *W. ugandensis* extracts.

3.2.5 Determination of MIC and effect of sub-optimum concentration of *W. ugandensis* on susceptibility profiles of test strains to conventional antibiotics.

The lowest concentration at which no colonies were observed was 47.5 µl for both the root and stem-bark. Thus the MIC was 42.5 µg/ml for both *W. ugandensis* methanolic root and stem-bark extracts. From the MIC values, the highest sublethal extract concentration for both the root and stem-bark was thus 32.5 µg/ml (Figure 7).

Bacteria strains were subjected to a sub-optimum concentration (32.0 µg/ml) of *W.ugandensis* methanolic extracts from the root and stem-bark. There were no significant differences in the antibiotic susceptibility profiles of the bacterial strains before and after habituation to sub-optimal extract concentration (t-test, $p>0.05$) as shown in Table 4. All the other clinical isolates had a similar trend.

Table 4: Mean inhibition zones of 15 conventional antibiotics against a *S. typhi* isolate, S3, before and after exposure to sub-lethal concentration of *W. ugandensis* methanolic root extract.

	Antibiotics														
	NOR	SMX	NA	TM	C	N	S	MN	CXM	CFM	CRO	ATM	FOX	AUG	AMP
Mean zones (mm) of isolate S_3 before exposure	23	11	13	10	12	15	9	15	12	18	14	14	15	9	8
Mean zones (mm) of isolate S_3 after exposure	22	12	14	9	13	16	10	16	13	17	15	16	16	8	6
t-test (p value)	0.03	0.14	0.27	0.13	0.96	0.21	0.45	0.35	0.56	0.92	0.91	0.61	0.9	0.17	0.16

Antibiotics:

NOR: Norfloxacin
SMX: Sulfamethoxazole
NA: Nalidixic acid
TM: Trimethoprim
C: Chloramphenical
N: Neomycin
S: Streptomycin
MN: Minocycline
CXM: Cefuroxime
CRO: Ceftriaxone
ATM: Aztreonam
FOX: Cefoxitin
AUG: Augmentin
AMP: Ampicilin

3.2.6 Effect of pH of diluents on inhibitory characteristics of *W. ugandensis*

The type and pH of the diluent used for dissolving the herbal extracts was found to influence inhibitory characteristics of the extracts. The inhibition characteristics of methanol extracts from the root preparation dissolved in deionised distilled water (pH 7), normal saline (pH 7) and TBE buffer (pH 8) were not significantly different (t-test, $p>0.05$). However, there was a significant difference between the inhibitory characteristics of the extracts dissolved in TBE pH 8 and those in pH 3 or 11 (t-test, $p<0.05$). Normal saline, distilled water and TBE pH 8 (containing no extract) did not inhibit bacterial growth. However, colony counts from preparations in TBE pH 3 and 11 were lower than those in TBE pH 8 indicating that this buffer could inhibit bacterial growth at acidic and alkaline pH even in the absence of plant extracts (Figure 8). The mean colony counts were obtained by averaging the colony counts for the duplicate sets at 50 µg/ml concentration of *W. ugandensis* methanolic root-bark extracts against a clinical *P. aeruginosa* isolate, P_3, and their standard error (SE) obtained (Figure 8)**.** Inhibition was similar for the other clinical isolates. Statistical data is shown in Appendix 6.

3.2.7 Analysis of interactive effects among extracts obtained from different parts of *W. ugandensis*

The methanol extracts from the root, stem-bark and leaf of *W. ugandensis* were assessed for synergistic interaction. The inhibitory characteristics of either the root or the stem-bark extracts were not significantly different from those obtained when the two extracts were mixed and used together (t-test, $p>0.05$) (Figure 9). However, the

inhibitory characteristics of the root /stem-bark combination was significantly higher than that of the root/leaf and stem-bark/leaf combinations (t-test, $p < 0.05$).

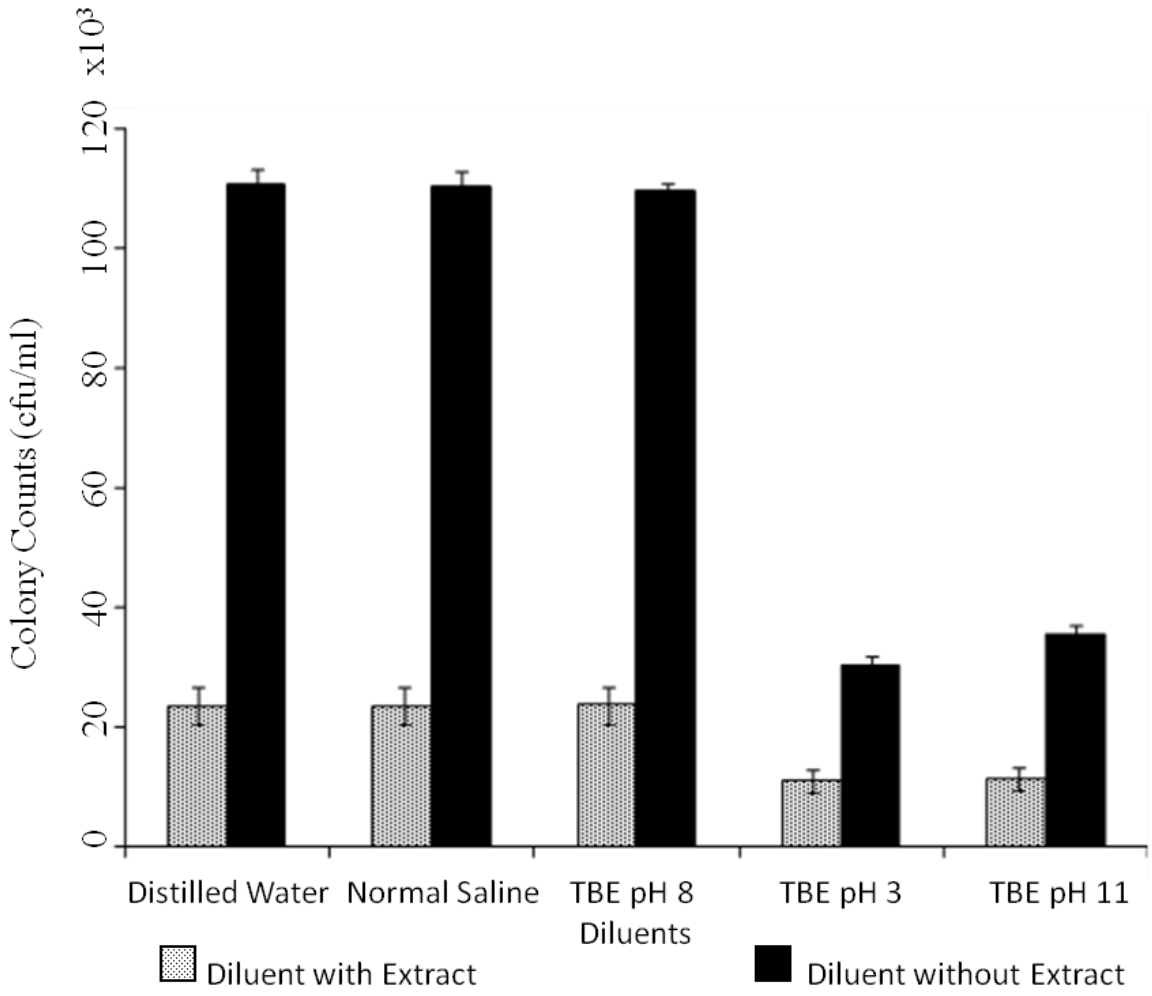

Figure 8: Inhibitory effect of root/stem-bark methanol extracts from *W. ugandensis* at different pH of the diluents against a clinical *P. aeruginosa* isolate, P_3.

The effectivity of the combined preparations from *W. ugandensis* was always lower than that obtained when root or stem-bark extracts were used separately. Thus, there was no synergy between the root, leaf and stem-bark extracts of *W. ugandensis.* The

mean colony counts were obtained by averaging the colony counts for the duplicate sets at 50 µg/ml concentration of *W. ugandensis* methanolic extracts against a clinical *K. pneumoniae* isolate, K_2, and their standard error (SE) obtained (Figure 9)**.** Inhibition trend was similar for the other clinical isolates.

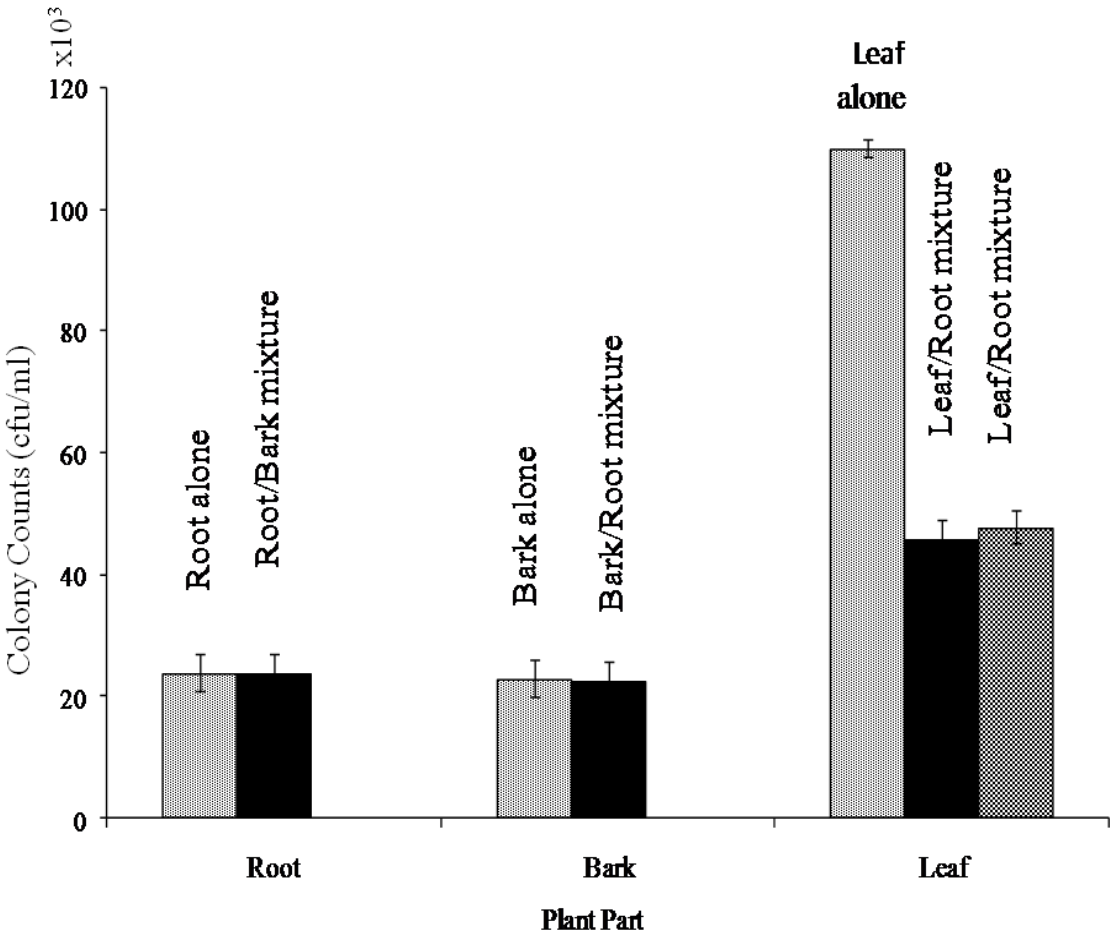

Figure 9: Interactive effect between methanol extracts obtained from different parts of *W. ugandensis* against a clinical *K. pneumoniae* isolate, K_2.

3.3 Effect of the herbal extracts on *in vitro* conjugation.

Only eight out of 27 donors transferred conjugative plasmids to the recipient *E. coli* J53 strain. The frequency of transfer, expressed as number of transconjugants per recipients, ranged between 2.0×10^6 and 4.0×10^6 with an average of 3.0×10^6 (Table 5). Eight donor strains transferred plasmids to the recipient *E. coli* J53 at the same frequencies in the presence of a sub-lethal amount of the extract. This implied that there was no reduction or increase in the transconjugant population when the conjugation cultures were incubated in the presence of sub-lethal amount of the extract. The sub-lethal extract concentration used was 32.5 µg/ml and colony counts were obtained from 1µl of total reaction volume.

There were significant differences in the antibiotic susceptibility profiles of the donors and transconjugants exposed to the stem-bark and root extracts (t-test, $p<0.05$) and those not exposed (t-test, $p<0.05$). However, there were no significant differences in the antibiotic susceptibility profiles of the exposed and non-exposed transconjugants (t-test, $p>0.05$), implying that exposure of the bacterial strains to sub-optimal extract concentration had no effect on their susceptibility to conventional antibiotics (Table 6). P value (t-test) indicates the differences between non-exposed and exposed donor strains; and non-exposed and exposed transconjugants.

Table 5: Conjugation frequencies for the transconjugants before and after exposure to sub-lethal concentration of *W. ugandensis* methanol root extracts.

Donor Strain	Mating frequencies before exposure	Mating frequencies after exposure
E_1	3.0×10^6	3.0×10^6
E_4	3.0×10^6	3.0×10^6
E_6	3.0×10^6	3.0×10^6
P_1	2.0×10^6	3.0×10^6
P_6	2.0×10^6	2.0×10^6
S_4	4.0×10^6	4.0×10^6
S_5	4.0×10^6	3.0×10^6
K_4	2.0×10^6	2.0×10^6

Donor strains: *E. coli* isolates: E_1, E_4 and E_6; *S. typhi* isolates: S_4 and S_5; *K. pneumoniae* isolate: K_4; *P. aeruginosa* isolates: P_1 and P_6.

Table 6: Inhibition zones for donor strains and their corresponding transconjugants before and after exposure to sub-lethal concentration of *W. ugandensis* methanolic root extracts.

Strains	Nor	SMX	NA	TM	C	N	S	MN	CXM	CFM	CRO	ATM	FOX	AUG	AMP	t-test
K_4	7	6	7	6	9	13	7	12	7	11	6	6	23	6	7	0.841
Ex- K_4	6	6	6	6	6	16	6	6	6	12	6	23	20	6	6	
t-K_4	25	6	22	8	25	18	9	13	6	21	9	12	29	9	6	0.367
t_E-K_4	23	6	21	7	24	17	8	13	6	20	8	12	28	8	7	
E_6	22	6	21	7	23	15	6	17	6	12	7	11	24	6	6	0.433
Ex- E_6	21	7	20	7	22	15	6	16	6	12	7	12	24	6	6	
t-E_6	27	6	20	6	28	18	15	19	6	13	9	12	29	9	6	0.298
t_E-E_6	26	7	21	7	27	17	14	18	8	14	8	15	27	7	7	
P_1	20	14	8	6	10	6	8	11	19	20	20	24	6	6	6	0.424
Ex- P_1	18	14	8	6	12	6	8	13	19	20	20	24	6	7	6	
t-P_I	35	6	19	6	6	17	6	15	20	29	31	34	18	6	6	0.465
t_E-P_1	30	8	19	7	6	17	7	14	19	28	28	32	18	6	7	
S_4	22	6	21	7	6	17	6	13	25	30	31	29	16	12	7	0.238
Ex- S_4	25	7	22	7	6	17	6	14	25	29	30	30	17	12	6	
t-S_4	30	6	24	6	6	17	6	16	19	30	34	31	18	6	6	0.652
t_E-S_4	31	6	23	7	6	18	6	16	18	30	31	31	18	6	6	
S_5	7	6	6	6	9	16	6	15	21	32	31	30	27	12	7	0.424
Ex- S_5	9	8	6	7	9	16	6	15	21	32	31	30	27	10	7	
t-S_5	6	24	22	6	6	10	20	17	30	34	36	32	30	11	6	0.500
t_E-S_5	6	24	21	6	7	10	21	17	29	31	29	32	30	12	6	
P_6	29	27	7	6	17	20	14	17	7	13	19	6	6	7	6	0.433
Ex- P_6	26	27	6	6	17	22	15	15	6	14	18	6	6	6	7	
t-P_6	30	6	24	6	25	16	6	18	6	15	10	16	28	7	8	0.125
t_E-P_6	30	6	24	6	25	16	6	17	6	14	10	16	25	7	8	
E_4	18	7	17	6	6	13	6	14	7	10	6	11	19	6	6	0.471
Ex- E_4	22	6	16	8	21	12	7	21	8	6	6	12	10	6	6	
t-E_4	30	6	25	6	6	20	6	18	30	30	32	34	29	7	6	0.245
t_E-E_4	29	7	24	7	6	20	7	17	29	29	29	31	23	6	7	
E_1	20	9	13	8	23	12	6	19	8	9	6	10	10	6	7	0.552
Ex- E_1	21	8	16	6	7	12	6	14	7	13	6	11	18	7	6	
t-E_1	30	6	26	6	26	16	6	22	26	31	33	34	22	11	6	0.524
t_E-E_1	30	7	23	7	25	15	7	23	24	27	28	31	20	13	7	

t: Transconjugant before exposure to sub-optimal extract concentration; t_E: Transconjugant after exposure to sub-optimal extract concentration. **Non-exposed donors**: *E. coli* isolates: E_1, E_4 and E_6; *S. typhi* isolates: S_4 and S_5; *K. pneumoniae* isolate: K_4; *P. aeruginosa* isolates: P_1 and P_6. **Exposed donors**: *E. coli* isolates: Ex-E_1, Ex-E_4 and Ex-E_6; *S. typhi* isolates: Ex-S_4 and Ex-S_5; *K. pneumoniae* isolate: Ex-K_4; *P. aeruginosa* isolates: Ex-P_1 and Ex-P_6.

CHAPTER FOUR

4.0 DISCUSSION

The 27 Gram-negative isolates used in this study were found to be resistant to various combinations of antibiotics including quinolones, cephalosporins, aminoglycosides, tetracyclines, trimethoprim, and some had resistance to β-lactam/β- lactamase inhibitors such as augmentin. Most of this resistance was shown to be transferable through conjugative plasmids. In Gram-negative bacteria, integrons and conjugative plasmids play an important role in dissemination of antimicrobial resistance genes.

The antibacterial activity observed for the methanol extracts from root and stem-bark of *W. ugandensis* against the MDR strains indicate their potential for further development of effective alternative antimicrobial remedies. *W. ugandensis* is used traditionally to treat bacterial infections and previous studies have reported its antibacterial activity. Phytochemical investigations of this plant indicate the presence of drimane-type sesquiterpenoids as the main active components (Brooks and Draffan, 1969; Wube *et al*. 2005). Known sesquiterpenoids from *W. ugandensis* include ugandensolide, ugandesidial, warburgin and warburgiadione commonly detected from the heartwood, while muzigadiolide, deacetylugandensolide, cinnamolide, mukaadial, ugandensidial, muzigadial and waburganal are isolated from the stem-bark. Flavonol glycosides and monoterpenes have also been detected from the leaves of this plant (Manguro *et al.* 2003). It is therefore possible that any of

these active compounds could be responsible for the antibacterial activities reported in the current study.

Crude extracts and purified compounds from *W. ugandensis* have been reported to be effective against *Mycobacterium tuberculosis*, *E. coli* and *Vibrio cholerae* (Mbwambo *et al.,* 2009). Previously, the stem-bark extracted using dichloromethane from this plant were examined for their antimycobacterial activity against *M. aurum, M. fortuitum, M. phlei* and *M. smegmatis* (Wube *et al.,* 2005). The active constituents showed MIC values ranging from 4 to128 µg/ml compared to the antibiotic drugs ethambutol (MIC range from 0.5 to 8 µg/ml) and isoniazid (MIC range from 1 to 4 µg/ml). This closely correlates with the results of our study in which the methanolic root and stem-bark extracts showed strong antibacterial activity with an MIC value of 42.5 µg/ml. Such an MIC indicates a strong potential for use of this plant as an antibacterial agent or detergent. In general, plant extracts are more effective against Gram-positive than against Gram-negative bacteria (Suffredini *et al.*, 2006). However, our study showed that the Gram-negative organisms used were sensitive to the plant extracts even at low MICs. Therefore, these findings further support the idea that *W. ugandensis* could be an important source of compounds with broad-spectrum antimicrobial properties.

The test strains used in this study were resistant to more than four classes of conventional antibiotics such as aminoglycosides, quinolones, cephalosporins and β-lactams among others. Some species including *K. pneumoniae, E. coli* and *P.*

aeruginosa are known to use efflux pumps to mediate resistance to antibiotics and other unrelated molecules. Considering that the extracts were effective against members of these species, the inhibitory efficiency of the root and stem-bark extracts from *W. ugandensis* is an important finding. The results further support the idea that the extracts are effective against both ATCC and MDR strains. The varied antibacterial activity between the root, stem-bark and leaf extracts may be attributed to different secondary metabolites in these plant parts. These extracts may offer less expensive treatment options of diseases caused by MDR strains. Characterization of the specific substance(s) conferring the antibacterial properties to the root-bark and stem-bark extracts will therefore be an important step for potential development of novel antimicrobial agents against related strains.

Previous studies by Matu and Staden (2003) reported that extracts of *A. remota* had no antibacterial activity on the test bacteria and this is in agreement with our findings in which case the multi-resistant Gram-negative strains of *E. coli*, *K. pneumoniae*, *P. Aeruginosa* and *S. typhi* were not inhibited. High antibacterial properties of *A. Secundiflora* have been reported (Waihenya *et al.,* 2002). The plant was however non-inhibitory in this study. *A. secundiflora* is known to contain only small amounts of saponins and sterols and this could partially be the reason for its poor antimicrobial activity. Our results for *Terminalia brownii* agree with the results by Mbwambo *et al.* (2007) who reported antibacterial activity of this plant against ATCC strains of *E. coli, P. aeruginosa, S. typhi,* and *K. pneumoniae*. A previous study by Thakurt *et al*. (2007) reported significant antibacterial activity of

Azadirachta indica leaf extracts against multi-drug-resistant *Vibrio cholerae.* However, in the present study, extracts from the plant were found to be non-inhibitory against the MDR clinical strains. This could be attributed to the different bioassay methods. In this study, all the extraction solvent (methanol) was fully evaporated and the pH of the eluting buffer (TBE pH 8) was also optimized. This was to avoid misinterpretation of results as both the extraction and eluting solvents could also inhibit bacterial growth.

The contrasting results of this study with those published by other investigators on antimicrobial activities of other plant extracts apart from *W. ugandensis* could be due to methodological differences. For instance, Thakurta *et al*. (2009) reported significant antibacterial activity of *Azadirachta indica* leaf extracts against multi-drug-resistant *Vibrio cholerae*, while Tajamul *et al*. (2010) reported good antibacterial activity of *Ricinus communis* against pathogenic bacterial strains like *K. pneumoniae* and *E. coli*. This could be attributed to differences in bioassay techniques used and diluents used for extraction and elution. Thakurt *et al*. (2007) and Tajamul *et al*. (2010) used the disc diffusion technique to assay the antimicrobial activity of the plant extracts. Such standard antimicrobial susceptibility testing methods like the agar diffusion and Kirby-Bauer may result in misinterpretation of results especially for extracts with low antimicrobial activity or in cases where the active ingredient(s) may irreversibly bind to the paper discs (Das *et al.,* 2010). In a previous study, Oyetayo (2008) showed that herbal remedies may not show any sign of inhibition of the bacteria when the agar diffusion method was used. To overcome

these drawbacks, the antimicrobial activity of the crude plant extracts was detected through a direct contact broth microdilution method. Inhibitory characteristics of the extracts were determined by performing viable colony counting following direct inoculation. This method allows for maximum action by the extract on the bacterial cells hence generating more reliable data. Besides the methodological approaches used by various investigators, the contrasting results could also be attributed to the locality of plant species, storage conditions, test strains used, and lack of appropriate positive and negative controls in some of these studies.

The use of potentially toxic solvents for extraction and elution requires that the solvent tolerance of the test strains be tested to establish the optimal concentration and pH values at which the solvents would result in significant reduction in viable cell counts. Good experimental designs ensuring that the extraction solvents such as methanol are fully evaporated in order to avoid misleading results. Evaporation conditions should also be controlled so as to minimise the chances of loosing the thermal-labile active ingredients. Similarly, the pH of the eluting buffers should be optimized to enhance their ability to dissolve the active ingredients in the plant extracts. However, if the potential toxicity of the eluting solvents is also not checked, the results could be misinterpreted. The higher potency observed in methanol extracts unlike the water extracts may be due to polarity of the solvents and their ability to better elute the active ingredients (Parekh *et al.,* 2005). It is known that highly polar solvents are able to extract phytochemicals efficiently and thus methanol or related organic solvents may be ideal for elution of active ingredients. These

differences in polarity determine the solubility of the extracts into the growth media although further characterization of the test extracts would be necessary to prove this hypothesis. Previous studies have shown that plant extracts in organic solvents like methanol provided consistent antimicrobial activity as compared to those extracted in water (Parekh *et al.,* 2005). Additionally, methanolic extracts from plants consistently provide more antimicrobial activity compared to those extracted in ethanol, or other more polar substances (Cowan, 1999). The higher anti-bacterial activity of methanol extracts is hypothesized to be due to the polarity of the solvent, and to the ability to dissolve or diffuse into the media used in the assays (Cowan, 1999). However, since methanol is harmful to eukaryotic cells, it is important to test other alternative solvent that may be used for extraction of phytochemicals with potential for *in vivo* use.

The bacterial resistance observed for other plant extracts (*T. brownii, A. indica, C. anisata, S. henningsii, Z. chalybeum, A. remota, T. indica, R. communis* and *A. secundiflora*) indicates that although they are traditionally used in the management of bacterial diseases, not all prescribed antibacterial medicinal plants may be effective against multi-drug resistant Gram-negative pathogens. A significant part of the chemical diversity produced by plants is thought to protect plants against microbial pathogens. Gibbons (2004), observes that a number of plant compounds often classified as antimicrobials produce MIC ranges greater than 1,000 µg/ml which are of no relevance from a clinical perspective. Tegos *et al.* (2002) suggests that a vast majority of plant compounds showing little *in vitro* antibacterial activity are not

antimicrobial but are regulatory compounds playing an indirect role in the plant defence against microbial infections. The results may also suggest that the resistance observed for other plant extracts may partially be due to efflux pumps in the bacteria. Many efflux systems are multidrug transporters capable of expelling a wide spectrum of structurally unrelated drugs and antimicrobials, thus contributing significantly to bacterial multidrug resistance (Veen and Konings, 2007). However, this study does not rule out the potential of such plants as agents for the treatment of infections caused by other agents. The plants may be effective while in combination with other medicinal plants or in combination with conventional antibiotics but further research to support this opinion is needed. Furthermore, some plants found not to have inhibitory effects *in vitro* may undergo enzymatical processing to make them effective *in vivo.* Others may become more potent in the presence of other components involved in immune response (Gonzalez-Lamothe *et al*., 2009).

The sesquiterpenoid farnesol was shown to drastically increase the susceptibility of Methicillin-Resistant *Staphylococcus aureus* (MRSA) toward β-lactams by specifically inhibiting the recycling of the C55 lipid carrier needed in peptidoglycan biosynthesis (Kuroda *et al.,* 2007). Such synergistic activities of herbal extracts obtained from different plants have not been fully investigated. This study attempted to investigate whether such synergistic activities was obtainable between extracts from different parts of *W. ugandensis*. While the combined effects of the mixture of natural compounds found *in planta* might be necessary to obtain a synergistic antibacterial activity against pathogenic bacteria, there was no synergy between the

root, leaf and stem-bark extracts of *W. ugandensis*. This may have been due to similar types of compounds in the different parts of the plant but in different concentrations. This could also suggest lack of chemical interaction between the antimicrobial compounds in the different plant parts or the ability of the Gram-negative MDR strains to circumvent the toxic effects of the plant metabolites.

A number of laboratory *in vitro* studies have demonstrated possible associations between the exposure of bacterial cultures to sub-effective concentrations of conventional antibiotics and changes in antibiotic susceptibility (Beumer *et al*., 2000). However, very little has been done to screen herbal extracts for similar effects and it is not known if such herbal-elicited antibiotic resistance is transferable via conjugation or whether the presence of herbal agents stimulate or inhibit conjugal transfer of antibiotic resistance. A study by McMahon *et al*. (2007) reported that the continued use of tea tree oil as a herbal remedy in sub-optimal concentrations may elicit the emergence of resistance to conventional antibiotics. Exposure of the bacterial strains to sub-optimal extract concentration of *W. ugandensis* extracts had no effect on their susceptibility to conventional antibiotics and conjugative efficiency. However, there is evidence that the transfer of conjugative plasmids between donor and recipient cells is significantly affected in the presence of other synthetic biocides such as cationic agents and organomerculials. Pearce *et al.* (2000) reported that sub-MIC concentrations of chlorhexidine, povidone iodine and cetrimide were able to increase conjugative transfer of resistance determinants. Equally, Christensen *et al*. (2001) found that conjugative transfer of the TOL plasmid

pWWO was high in the presence of synthetic phenol within a three-species biofilm community.

Currently, no herbal product has been approved for systemic use to combat bacterial infections because the mode of action of purified components is not well defined. It has also been difficult to isolate specific antimicrobial compounds from plant extracts consisting of a mixture of a large number of structurally unrelated compounds with varying degrees of bioactivity or in other cases, the active ingredient is found to be less effective that the total crude extract (Jaki *et al.*, 2008). Thus, this study did not attempt to isolate the active ingredients and the herbal extracts were screened as crude extracts derived using methanol and water extraction.

CONCLUSIONS

1. The 27 the test strains were multi-drug resistant as they were resistant to more than four classes of conventional antibiotics including aminoglycosides, quinolones, cephalosporins and β-lactams among others. This resistance was mediated by class 1 integrons, *bla-*$_{SHV}$ and *bla-*$_{TEM}$ genes and was also transferable through conjugative plasmids.

2. Methanol extracts from root-bark and stem-bark of *W. ugandensis* exhibited better antimicrobial properties than the other nine plant extracts tested therefore supporting the rationale for use in traditional treatment of diarrhoea, stomach

aches and other bacterial infections. However, a combination of extracts from the same plant is not synergistic.

3. The MIC of methanol extracts from the root-bark and stem-bark of *W. ugandensis* against the MDR gram-negative bacterial strains was 42.5 µg/ml. The MIC value of the active extracts is within a comparable range to that of conventional antibiotics suggesting that such extracts may provide superior sources of bioactive compounds with activity on MDR strains.

4. Conjugation experiments indicate that even though the methanol extracts from the root and bark of *W. ugandensis* were effective against the MDR strains, they neither stimulated nor inhibited the transfer of resistance determinants in bacteria at sub-lethal concentrations.

5. Sub-lethal extract concentrations of the effective extracts of *W .ugandensis* do not affect the sensitivity profiles of the test strains to conventional antibiotics. This further indicates that extracts from these plants may not jeopardize the use of conventional antibiotics.

In respect to the findings above, the null hypothesis is rejected. This is because methanol extracts from the root-bark and stem-bark of *W. ugandensis* extracts were inhibitory to growth of the clinical MDR Gram-negative bacterial strains.

RECOMMENDATIONS

On the basis of zones of inhibition, this study revealed that extracts from five plants inhibited the growth of *E. coli* strain J53 indicating that such plants have potential as antimicrobial agents and may therefore require further testing for drug development. The findings of this study that the MDR strains were only inhibited by methanol preparations from root and stem-bark of *W. ugandensis* indicate that the extracts provide potential sources of antimicrobial compounds effective against MDR strains. Therefore, further studies are needed to isolate and characterize the specific active constituents in the root-bark and stem-bark extracts of *W. ugandensis* conferring the antibacterial properties in order to obtain safe antimicrobial products in the form of chemotherapeutic agents or antiseptics.

The ethno-botanical study of a plant is important for modern day medicine but its usefulness cannot be overemphasized if methods are not standardized to obtain comparable and reproducible results. Thus, it is important to standardize methods of extraction and *in vitro* antimicrobial efficacy testing so that the search for new biologically active plant products could be more systematic and interpretation of results would be facilitated.

Further tests are required to investigate the synergistic effect of extracts from *W, ugandensis*. In addition, further tests to investigate the *in vivo* interactions of components from this plant with antibiotics need to be done. Also, the effect of active compounds on beneficial and normal microbial flora in the human body would

have to be determined as well as an analysis of the risk/benefits of potential application in humans, including toxicity studies. Thus, efficient collaborations with pharmacologists and medical doctors, plant pathologists and microbiologists are crucial to see the complete development of exploitable effective antimicrobial products from *W. ugandensis* that are active against multi-drug resistant bacteria. These antimicrobial products may be in the form of disinfectants for topical applications or as antimicrobials for *in vivo* use.

The potential toxicity of herbal extracts is an important consideration when studying the use of plant extracts as alternative antimicrobial remedies. Thus, in determining the safety of root-bark and stem-bark extracts for *W. ugandensis* as herbal products for approval, further tests are required to investigate effect of sub-lethal concentrations of the extracts on the sensitivity profiles to conventional antibiotics and conjugal transfer of resistance determinants in bacteria over a longer period of time. Additionally, extensive *in vitro* and *in vivo* toxicological studies and animal assays need to be undertaken possibly using more appropriate eukaryotic models. However, in the absence of such tests, such extracts may be used as detergents or antiseptics for cleaning surfaces or for topical applications in the control of bacterial proliferation.

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APPENDICES

APPENDIX 1: Comparison of inhibitory characteristics of different plants on clinical MDR strains

				95% Confidence Interval of the Difference				
Plant extracts	Mean	Std. D	S. E. Mean	Lower	Upper	t	df	Sig
Warbugia vs*Terminalia*	-31.977	50.284	1.814	-35.538	-28.415	-17.623	767	.000
Warbugia vs *Azaridachta*	-32.630	50.617	1.826	-36.216	-29.045	-17.865	767	.000
Warbugia vs *Clausena*	-32.268	49.974	1.803	-35.808	-28.728	-17.894	767	.000
Warbugia vs *Strychnos*	-32.415	49.918	1.801	-35.951	-28.879	-17.996	767	.000
Terminalia vs *Azaridachta*	-.654	16.876	.609	-1.849	.542	-1.073	767	.283
Terminalia vs *Clausena*	-.292	18.338	.662	-1.591	1.007	-.441	767	.659
Terminalia vs *Strychnos*	-.439	18.110	.653	-1.722	.844	-.671	767	.502
Azaridachta vs *Clausena*	.362	19.609	.708	-1.027	1.751	.512	767	.609
Azaridachta vs *Strychnos*	.215	19.478	.703	-1.165	1.595	.306	767	.760
Clausena vs *Strychnos*	-.147	18.390	.664	-1.450	1.156	-.222	767	.825

APPENDIX 2: Comparison of inhibitory characteristics of different parts of *W. ugandensis* on clinical MDR strains

				95% Confidence Interval of the Difference				
	Mean	Std. D	S E Mean	Lower	Upper	t	df	Sig
Bark methanol vs Bark water	-85.079	36.252	3.217	-91.445	-78.713	-26.448	126	.000
Root methanol vs Root water	-63.898	55.239	4.882	-73.560	-54.237	-13.087	127	.000
Leaf methanol vs Leaf water	2.117	22.942	2.028	-1.895	6.130	1.044	127	.298
Bark methanol vs Root methanol	-.126	12.236	1.086	-2.275	2.023	-.116	126	.908
Bark methanol vs Leaf methanol	-86.087	39.345	3.491	-92.996	-79.177	-24.657	126	.000
Root methanol vs Leaf methanol	-86.039	39.013	3.448	-92.863	-79.215	-24.951	127	.000
Bark water vs Root water	21.195	46.581	4.117	13.048	29.343	5.148	127	.000
Root water vs Leaf water	-20.023	49.360	4.363	-28.657	-11.390	-4.590	127	.000
Bark water vs Leaf water	1.172	18.302	1.618	-2.029	4.373	.724	127	.470
Bark methanol vs Root water	-63.669	54.424	4.829	-73.226	-54.112	-13.184	126	.000
Bark methanol vs Leaf water	-83.969	38.935	3.455	-90.806	-77.131	-24.304	126	.000
Root methanol vs leaf water	-83.922	39.161	3.461	-90.771	-77.072	-24.245	127	.000

APPENDIX 3: Inhibitory characteristics of increasing extract concentrations from different plants on clinical MDR strains

		Sum of Squares	df	Mean Square	F	Sig
Warbugia * titres	Between Groups (Combined)	103270.5	7	14752.92	6.661	0.00
Terminalia * titres	Between Groups (Combined)	246.286	7	35.184	0.266	0.967
Azaridachta * titres	Between Groups (Combined)	1257.792	7	179.685	0.87	0.53
Clausena * titres	Between Groups (Combined)	2375.578	7	339.368	1.736	0.097
Strychnos * titres	Between Groups (Combined)	1094.967	7	156.424	0.844	0.551

APPENDIX 4: Inhibitory characteristics of increasing concentrations for *W. ugandensis* stem-bark extracts

				95% Confidence Interval of the Difference				
Extract titres	Mean	Std. D	S E Mean	Lower	Upper	t	df	Sig
5 µl vs 10 µl	32.56250	16.43966	4.10992	23.80242	41.32258	7.923	15	.000
10 µl vs 27.5 µl	38.93750	34.73417	8.68354	20.42897	57.44603	4.484	15	.000
27.5 µl vs 32.5 µl	12.62500	29.73858	7.43465	-3.22157	28.47157	1.698	15	.110
32.5 µl vs 42.5 µl	4.12500	3.20156	.80039	2.41901	5.83099	5.154	15	.000
42.5 µl vs 47.5 µl	4.25000	1.77012	.44253	3.30677	5.19323	9.604	15	.000
47.5 µl vs 50 µl	.25000	.93095	.23274	-.24607	.74607	1.074	15	.300
50 µl vs 55 µl	.26667	.45774	.11819	.01318	.52015	2.256	14	.041

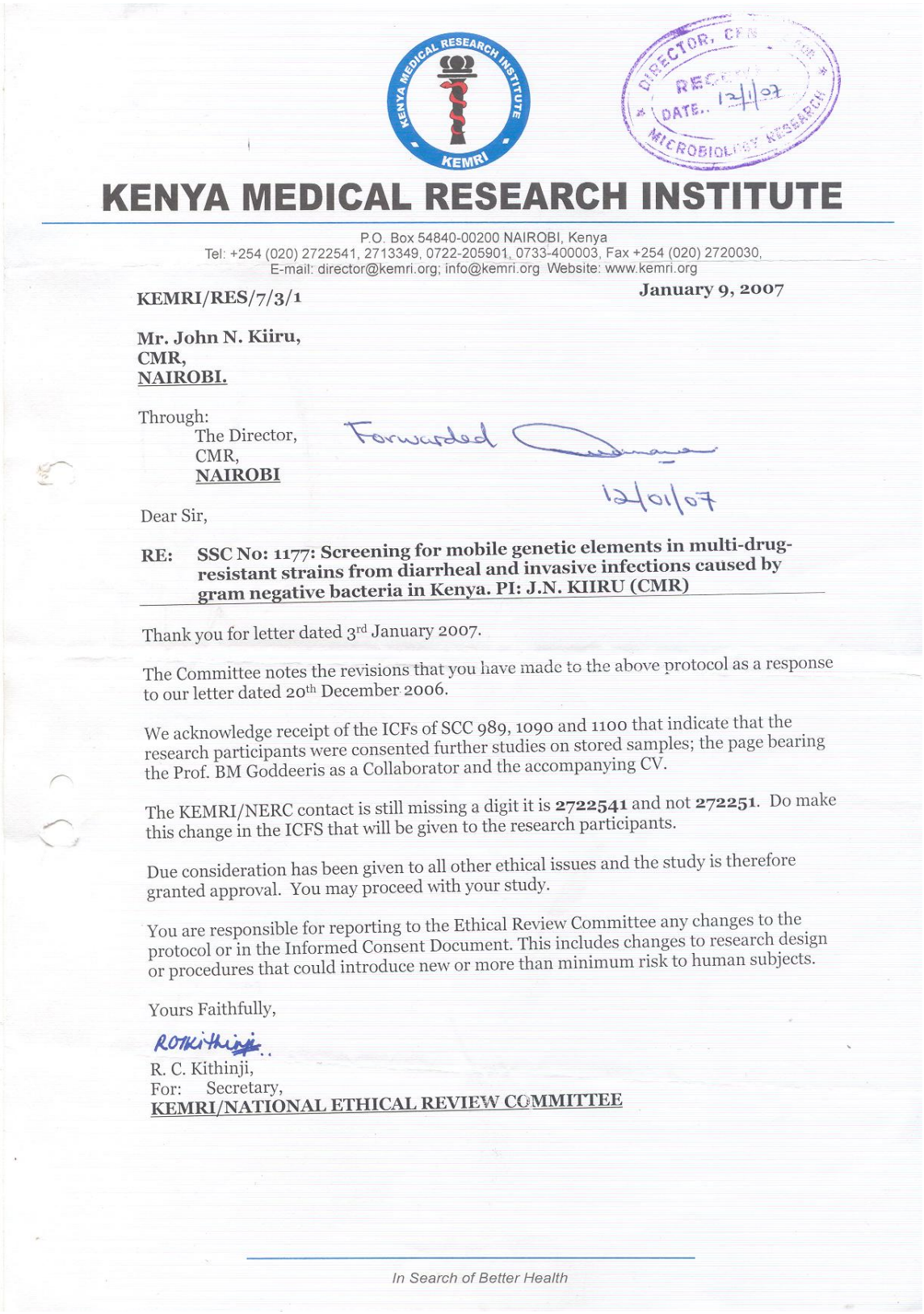
APPENDIX 5: Inhibitory characteristics of increasing concentrations for *W. ugandensis* root-bark extracts

				95% Confidence Interval of the Difference				
Extract titres	Mean	Std. D	S. E. Mean	Lower	Upper	t	df	Sig
5 µl vs 10 µl	35.81250	19.44297	4.86074	25.45207	46.17293	7.368	15	.000
10 µl vs 27.5 µl	45.31250	15.17330	3.79333	37.22722	53.39778	11.945	15	.000
27.5 µl vs 32.5 µl	7.56250	5.86480	1.46620	4.43737	10.68763	5.158	15	.000
32.5 µl vs 42.5 µl	6.18750	3.42965	.85741	4.35997	8.01503	7.216	15	.000
42.5 µl vs 47.5 µl	2.68750	1.30224	.32556	1.99358	3.38142	8.255	15	.000
47.5 µl vs 50 µl	.50000	.81650	.20412	.06492	.93508	2.449	15	.027

APPENDIX 6: Comparison of effect of pH of diluents on inhibitory characteristics of *W. ugandensis* root-bark extracts

Diluents	Mean	Std. D	S. E Mean	95% Confidence Interval of the Difference		t	df	Sig
				Lower	Upper			
Distilled water vs Normal saline	0.04	.34	.030	-.025	.09878	1.294	127	.198
Distilled water vs TBE buffer (pH 8)	-0.10	.66	.059	-.21737	.01425	-1.735	127	.085
Distilled water vs TBE buffer (pH 3)	12.58	19.39	1.71	9.18691	15.96934	7.340	127	.000
Distilled water vs TBE buffer (pH 11)	12.20	18.99	1.68	8.87223	15.51840	7.262	127	.000
Normal saline vs TBE buffer (pH 8)	-0.14	0.74	0.07	-.26998	-.01127	-2.151	127	.033
Normal saline vs TBE buffer (pH 3)	12.54	19.30	1.71	9.16428	15.91385	7.352	127	.000
Normal saline vs TBE buffer (pH 11)	12.16	18.92	1.67	8.84680	15.46570	7.269	127	.000
TBE buffer (pH 8) vs TBE buffer (pH 3)	12.68	19.12	1.69	9.33508	16.02429	7.502	127	.000
TBE buffer (pH 8) vs TBE buffer (pH 11)	12.30	18.79	1.66	9.01043	15.58332	7.404	127	.000
TBE buffer (pH 3) vs TBE buffer (pH 11)	-.38	6.31	.56	-1.48707	.72145	-.686	127	.494

APPENDIX 7: KEMRI ETHICAL REVIEW CLEARANCE LETTER



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KEMRI/RES/7/3/1

January 9, 2007

Mr. John N. Kiiru,
CMR,
NAIROBI.

Through:
The Director,
CMR,
NAIROBI

Forwarded
12/01/07

Dear Sir,

RE: **SSC No: 1177: Screening for mobile genetic elements in multi-drug-resistant strains from diarrheal and invasive infections caused by gram negative bacteria in Kenya. PI: J.N. KIIRU (CMR)**

Thank you for letter dated 3rd January 2007.

The Committee notes the revisions that you have made to the above protocol as a response to our letter dated 20th December 2006.

We acknowledge receipt of the ICFs of SCC 989, 1090 and 1100 that indicate that the research participants were consented further studies on stored samples; the page bearing the Prof. BM Goddeeris as a Collaborator and the accompanying CV.

The KEMRI/NERC contact is still missing a digit it is **2722541** and not **272251**. Do make this change in the ICFS that will be given to the research participants.

Due consideration has been given to all other ethical issues and the study is therefore granted approval. You may proceed with your study.

You are responsible for reporting to the Ethical Review Committee any changes to the protocol or in the Informed Consent Document. This includes changes to research design or procedures that could introduce new or more than minimum risk to human subjects.

Yours Faithfully,

R.C.Kithinji

R. C. Kithinji,
For: Secretary,
KEMRI/NATIONAL ETHICAL REVIEW COMMITTEE

In Search of Better Health