SCREENING OF ANTIDIARRHOEA MEDICINAL PLANTS FOR IN-VITRO ANTIMICROBIAL ACTIVITY AGAINST CLINICAL AND ENVIRONMENTAL ENTEROPATHOGENS

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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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I dedicate this work to my parents, Dr. and Mrs. Jotham Akanga for their continued support both emotionally and financially. May the fruits of my labour bring you all joy and happiness.

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ABBREVIATIONS

ACB:	*Albizia coriaria* Bark
ASL:	*Aloe secundiflora* Leaves
ASW:	Artificial Sea Water
ATCC:	American Type Culture Collection
BST:	Brine Shrimp Lethality Test
CFU:	Colony Forming Unit
DMSO:	Dimethyl-Sulfoxide
FL:	Fidelity level
KEMRI:	Kenya Medical Research Institute
MBC:	Minimum Bactericidal Concentration
MDR:	Multi-Drug Resistance
MHA:	Mueller-Hinton Agar
MHB:	Mueller-Hinton Broth
MIC:	Minimum Inhibitory Concentration
MVB:	*Melia volkensii* Bark
MVL:	*Melia volkensii* Leaves
NCCLS:	National Committee for Clinical Laboratory Standards
TBB:	*Terminalia brownii* Barks
TLC:	Thin Layer Chromatography
WHO:	World Health Organization

ABSTRACT

Plants in traditional medicine have been widely used to treat diarrhoea diseases in Kenya. Ethnobotanical surveys are useful in the identification and selection of medicinal plants with potential therapeutic values. Since no ethnobotanical study has been conducted in Rachuonyo district of Nyanza province to identify the plants commonly used for the treatment of diarrhoea, this study interviewed 191 respondents and found that *Terminalia brownii* Fres. (barks), *Melia volkensii* Guerke (barks), *Melia volkensii* Guerke (leaves), *Aloe secundiflora* Engl. and *Albizia coriaria* Welw. ex Oliv. (barks) were most frequently used. The plants were collected and extracted using hexane, methanol, acetone and water and tested against clinical and environmental enteric pathogens. Phytochemical tests indicated that the plants contained tannins, triterpenoids, flavonoids, steroids, alkaloids, glycosides, phenols and saponins in varying amounts. The antimicrobial assay of the plant extracts showed that the highest (24 mm zone of inhibition) activity was by methanol extracts of *Terminalia brownii* barks against *Vibrio cholerae* (clinical isolate) while acetone extracts of *Melia volkensii* leaves had the least activity (7 mm) against *Shigella dysenteriae* (clinical isolate). The extracts were active at relatively low concentrations, with their minimum inhibitory concentrations ranging from 3.13 mg/ml for *Melia volkensii* bark against *Shigella dysenteriae* to 50 mg/ml for *Albizia coriaria* bark against *E.coli* ATCC 25922. However, some of the plant extracts (*Aloe secundiflora*) were inactive against

some of the test isolates, indicating that not all prescribed antidiarrhoeal plants may be effective against enteric pathogens. The present study showed synergism between ampicillin, cefuroxime, gentamicin and cotrimoxazole with all plant extracts tested at various concentrations against some antibiotic-resistant bacteria (*V. cholerae, S. dysenteriae, Enterobacter aerogenes, Proteus mirabilis, Pseudomonas aeruginosa* and *Escherichia coli*). This indicates that the use of plant extracts together with antibiotics may enhance activity against drug-resistant pathogens. In the kill kinetics tests, the extracts of *Terminalia brownii* were bactericidal against *Staphylococcus aureus* while *Melia volkensii* and *Albizia coriaria* were bactericidal against *Escherichia coli*. This is interesting because *S. aureus* has been known to be therapeutically problematic especially in immunocompromised people. Hence, *Terminalia brownii* can be used in treating *S. aureus* infections in this group of people. The brine shrimp lethality tests revealed that the plants were of low toxicity and can be used for the treatment of diarrhoea diseases in humans and avoid possible detrimental health risks. The overall results of the present study authenticate the therapeutic values of the antidiarrhoeal medicinal plants and show that they can be used in further drug development.

CHAPTER 1

1.0 INTRODUCTION

Diarrhoeal diseases are still among the leading causes of mortality in developing countries, despite advances in understanding and management that have occurred over the years (WHO, 1996). It may result from infections such as cholera and dysentery, which come from the use of and contact with polluted water as well as from eating soil by pregnant women (Sindiga *et al*., 1995). Specific enteric bacteria are responsible for acute and persistent diarrhoea as well as dysentery. They are widely distributed in plants, in soil and in the intestines of humans and animals (Kelly *et al*., 1985). The most common etiology is *Escherichia coli*. Other enteric pathogens are species of *Salmonella*, *Shigella*, *Klebsiella*, *Enterobacter*, *Proteus* and *Yersinia*. *Klebsiella* and *Proteus* also cause pneumonia, ear, sinus and urinary tract infections. *Enterobacter* and *Serratia* often cause bacteremia. Those associated with diarrhoea include *Citrobacter, Proteus, Morganella, Hafnia, Edwardsiella, Enterobacter, Serratia* and strains of *Pleisomona shigelloides* (Saidi, 2004).

The presence of enteric pathogens in surface waters has public health implications. In many developing countries, with inadequate sanitation, fecal contamination of environmental waters by enteric pathogens is very common. Isolation of enteric bacteria from environmental sources is essential to characterize their pathogenic potential as well as their sensitivity to antimicrobial

agents (Faruque *et al*., 2002). A study using reports of food-borne outbreaks in the USA suggests that many infants acquire non-typhoidal salmonellosis from environmental sources other than food (Haddock, 1993). Faruque *et al*., (2002) analysed rRNA gene restriction patterns (ribotypes) and showed that the environmental isolates shared ribotypes with a collection of clinical isolates. The study also showed that most of the environmental strains were resistant to one or more antibiotics. This shows that the strains may also serve as reservoirs for drug resistance genes, hence posing a serous public health problem.

The World Health organization (WHO, 1964) constituted a Diarrhoeal Disease Control Program (DDC), which included studies of traditional medical practices, together with the evaluation of health education and prevention approaches (Devi *et al*., 2002). Medicinal plants represent a vast untapped source of medicines and have enormous potential for developing antimicrobial agents based on their indigenous and local knowledge. Further exploration of plant-based antimicrobials is necessary so that those plants that have shown promising antibacterial activity can be investigated and subsequently lead to development of new antibacterial agents from local less expensive sources.

Medicinal plants are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic drugs (Kone *et al*., 2004). The synthetic drugs are sometimes associated

with adverse side effects on some hosts such as hypersensitivity, immune-suppression and allergic reactions (Ahmad *et al*., 1998**).** Recently, multiple drug resistance has developed due to the indiscriminate use of synthetic antimicrobial drugs (Davis, 1994). In addition, bacteria have evolved numerous defenses against the antimicrobials (Ahmad and Aqil, 2006). Combination of these factors has created the need to search for alternative antimicrobial drugs from medicinal plants.

Basic information that may lead to scientific probing of medicinal plants in Africa is obtained from herbalists or traditional medicinal practitioners, herbal products vendors and the local people (Elujoba *et al*., 2005). Traditional medicine has contributed to development of new drugs that have helped in the dramatic decline in mortality, increase in life expectancy and reduction of morbidity due to diseases (Houghton, 1995). Many plants that provide active ingredients for prescription drugs came to the attention of researchers because of their use in traditional medicine. Some plants that have been used to develop commercial drugs include: *Atropa belladonna* L. (Solanaceae) which contains the alkaloid atropine, *Claviceps purpurea* (Fr.) Tul., which contains several toxic alkaloids like ergometrine that reduces blood loss after birth by contracting the muscles of the uterus and ergotamine that counteracts migraine. by constricting the small blood vessels. The drug digitalin, used to treat dropsy, was obtained from *Digitalis purpurea* L. while *Tamarindus indica* L., which contains tannins, helps

treat diarrhoea and dysentery (Sindiga *et al*., 1995). This study may provide new leads for the development of new antimicrobials that can be used to treat diarrhoea (due to antibiotic-resistant bacteria) in developing countries.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Enteric pathogens

Human faeces are the primary source of diarrhoea pathogens (WHO, 2000). The true pathogenic enterics include: *Salmonella typhi, Salmonella cholera-suis, Salmonella enteritidis, Shigella dysenteriae, Shigella flexneri, Shigella boydii, Shigella sonnei, Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. Specific pathogens cause acute diarrhoea, dysentery and persistent diarrhoea but the most important ones are enteropathogenic *Escherichia coli* and enteroaggregative *E. coli* (Ochoa *et al*., 2004). *E. coli* is the most prevalent enteric bacillus in clinical specimens and in infections due to its being the most common aerobic and non-fastidious bacterium in the gut. In developing countries like Kenya, the bacterial pathogens most commonly associated with endemic forms of diarrhoea are diarrhoeagenic *E. coli, Salmonella spp., Shigella, Vibrio cholerae, Aeromonas,* and *Pleisomonas spp.* (Mamtha, 2005).

2.2 Diarrhoea: A global and national concern

Diarrhoea is an acute syndrome of the intestinal tract in which the volume, fluid content and frequency of bowel movements increase (Talaro, 2005). The causative agents are transmitted through the oral-fecal route. Some of the factors that contribute to the frequency of occurrence of diarrhoea include poverty, which is associated with poor sanitation, lack of access to sufficient clean water and

appropriate sewage disposal (WHO, 1997; WHO, 2000). In the 21st century, diarrhoeal diseases continue to be a major cause of morbidity and mortality worldwide (O'Ryan *et al*., 2005). Recurrent diarrhoea is prevalent in developing countries, particularly in tropical regions (Pickering, 2004). Enteric pathogens are the most frequent causes of diarrhoea illness, which account for an annual mortality rate of 3 million and an estimated 4 billion infections worldwide (Talaro, 2005). There are three major diarrhoea syndromes that are produced by enteric pathogens: acute watery diarrhoea, persistent diarrhoea and bloody diarrhoea.

Diarrhoeal diseases are among the most common communicable diseases in Kenya and many parts of the world. They cause 4% of all deaths and 50% of health loss to disability. In South East Asia and Africa, it is responsible for as much as 8.5% and 7.7% of all deaths respectively. In 1998, diarrhoea was estimated to have killed 2.2 million people in Africa, most of who were under 5 years of age (WHO, 2000). In Kenya, diarrhoea diseases are among the top five major causes of mortality in children under five years. Infants are especially vulnerable to diarrhoea illness because of their smaller fluid reserves and underdeveloped immunity. Current estimates from show that Kenya has an annual incidence of between 3.5 and 4.6 diarrhoea episodes per child (PSI/Kenya, 2006).

2.3 Factors favouring the spread of diarrhoea in Kenya

In 1997, five districts of Nyanza province and parts of Western Kenya were struck by cholera (IFRC, 1997). Information from the International Federation's Regional Delegation in Nairobi confirmed official hospital data showing 2,500 registered cases with a 5-10% mortality rate in Nyanza and Western Kenya, due to cholera. Factors that contributed to the spread of the disease included the prevailing poor environmental and personal hygiene conditions, the common use of unsafe drinking water. Other factors include the onset of seasonal rains like *El Nino*, some traditional cultural practices (i.e. communal meals during funerals) and poor health infrastructure (IFRC, 1997). Most of the people in rural Nyanza obtain water from unprotected sources such as rivers. They mostly use pit latrines, which may be a source of fecal contamination of water from the nearby wells.

The lack of clean water in developing countries such as Kenya is responsible for billions of cases of diarrhoea that kills about 2 million children each year. Most enteric pathogens that are responsible for diarrhoea are spread by contaminated water (WHO, 2000). The microbial content of drinking water must therefore be continuously monitored to ensure that the water is free from infectious agents. Some of these agents can survive in natural waters for long periods without a human host (Talaro, 2005).

2.4 Treatment of Enterobacterial infections

Antimicrobial agents are administered to patients with diarrhoea caused by selected bacterial and protozoal pathogens to reduce signs, symptoms and duration of disease. They also prevent morbidity and mortality; eradicate fecal shedding of the causative organism and eliminate transmission (O'Ryan *et al*., 2005). First line antimicrobial drugs of choice for most Enterobacterial infections have been sulfonamides, tetracycline, ampicillin, trimethoprim-sulfamethoxazole, nalidixic acid and pivmecillinam. Resistance to these drugs has developed and presently, flouroquinolones are the only drugs that are effective (Frey, 2001). The standard anti-diarrhoea agents such as diphenoxylate, loperamide and deodorized tincture of opium have limited antimicrobial effects, and may cause significant central nervous system depression. This has led to patients turning to herbal medicines, which are affordable and show significant activity towards treatment of diarrhoea (Cohen *et al*., 2000).

Herbal medications are often high in tannins, which act as astringents and thereby act to control diarrhoea, and also reduce or prevent the colonization of the enteric pathogens (Lewis, 2003). They denature or precipitate proteins, thereby altering the surface structures of the enteric pathogens. Therefore, herbal therapy is recommended for stopping diarrhoea as well as treating the underlying causes (van Wyk, 2002). Some East African plants have been studied and shown significant inhibitory activity against enteric pathogens. These include fruits of

Acacia nilotica L. (Khan *et al*., 1980), stem bark of *Lannea stuhlmannii* Engl. (Chhabra, *et al*., 1987), and roots of *Dichrostachys cinerea* L. (Chhabra, *et al*., 1990), among others. In Kenya, some of the medicinal plants that are used in various communities include stem of *Adenia gummifera* Harv., bark of *Bridelia micrantha* (Hochst) Baill., fruit extract of *Rhus vulgaris* Meikle., and crushed plant of *Spilanthes mauritiana* Rich. Ex. Pers., among others (Kokwaro, 1976).

2.5 Resistance patterns among enteric pathogens

2.5.1 Resistance patterns among enteric pathogens in developing countries

Successful antibiotic treatment in developing countries has become problematic because of the increasing prevalence of antimicrobial drug resistance (Salam, 1998). A well-documented risk factor for developing infection with resistant bacterial pathogens is the recent use of antibiotics, particularly within 4 weeks before exposure to the pathogens (Pickering, 2004). In many countries of the world, an increase in antibacterial resistance patterns has occurred among the major bacterial pathogens, including *Shigella* spp., *E. coli* pathotypes associated with diarrhoea, *Mycobacterium tuberculosis*, *Campylobacter jejuni/coli, Vibrio cholerae*, non-typhoidal *Salmonella* and *Salmonella* spp. Some of them are acquiring resistance to several classes of antimicrobial agents (Okeke *et al*., 1999; O'Ryan *et al*., 2005).

The progressive increase in antimicrobial resistance among enteric pathogens especially in *Shigella*, *V. cholerae*, Enterotoxigenic *E. coli* and *S. typhi* is becoming a critical concern for the people of the developing world, where there are high rates of diarrhoea diseases and associated mortality (Sack *et al*., 1997; Guervero, 2001). There is a variation of resistance patterns within countries in the East African region. In a study carried out between 1994 and 1996, 80-100% of isolates of *V. cholerae* from Kenya and Southern Sudan, and 65-90% from Somalia were sensitive to tetracycline. All isolates from Tanzania and Rwanda were 100% resistant to tetracycline (Materu *et al*., 1997). The same study also indicated that in Kenya and Somalia, the percentage of the isolates sensitive to chloramphenicol and cotrimoxazole reduced markedly from 85% in 1994 to less than 10% in 1996. All of the isolates from Rwanda and Tanzania were resistant to chloramphenicol and cotrimoxazole while in Southern Sudan, more than 70% of the isolates were sensitive. *Sh. dysenteriae* and *Sh. flexneri* showed similar antibiotic sensitivity patterns and were sensitive only to nalidixic acid and furazolidone (Materu *et al*., 1997). The only effective antibiotics to enteric pathogens are the newer flouroquinolones (Bennish, 1995) such as ciprofloxacin and levofloxacin. However, increasing microbial resistance to the flouroquinolones may limit their usefulness in some geographical areas such as Thailand and Nepal. In such cases, azithromycin and rifaximin can be used as alternatives (CDC, 2007).

The emergence and spread of the resistant enteric organisms occurs because of mutation in common resistance genes, exchange of genetic information among microorganism, and climate change (Saidi, 2004). It may also be due to spread of multiple resistant bacterial clones and selective pressure of antibiotic therapy in communities and hospitals that facilitate development and spread of resistance (Joel *et al*., 1996; Patrick *et al*., 1999; Tenover, 2001). Mobile genetic elements e.g. plasmids and transposons have been implicated in the spread of antibiotic resistance genes between bacteria (Talaro, 2005). Some factors responsible for the persistence of antibiotic resistance among bacteria include the presence of enzymes within the cell surface that inactivate drugs or the possession of impermeable cell membranes that prevent influx of drugs (Joel *et al*., 1996).

In developing countries, antimicrobial resistance is most likely related to the frequent unrestricted use of over-the-counter drugs without medical supervision and inclusion of various classes of antimicrobial agents as growth promoters in feeds of livestock (Guervero, 2001). People infected with enteric pathogens that are resistant to frequently used antimicrobial agents may manifest as either clinical or bacteriologic treatment failures and may have an extended duration of excretion of viable organisms. Gram-positive bacteria freely transfer chromosomal or plasmid-borne genes that can mediate drug resistance. When this occurs in mixed populations of fecal residents such as in the intestines, it is

possible for non-pathogenic or less pathogenic residents to acquire greater virulence or resistance (O'Ryan *et al*., 2005)

2.5.2 Resistance patterns among enteric pathogens in Kenya

Despite the problem of rapid increase in antibiotic resistance in Kenya, not much is known about the epidemiology and resistance patterns of most enteric bacterial pathogens. Few studies have been carried out in Kenya on antimicrobial resistance of enteric pathogens (Materu *et al*. 1997; Kariuki *et al*., 2000; Ndung'u *et al*., 2004; Saidi, 2004; Brooks *et al*., 2006; Scrascia *et al*., 2006). In 1998-1999, there was the history of the largest cholera epidemic in Kenya. Of the 80 *V. cholerae* 01 strains that were selected for the study of the epidemic, 61 strains were resistant to chloramphenicol, spectinomycin, streptomycin, sulfamethoxazole and trimethoprim (Scrascia *et al*., 2006). Resistance to the drugs of choice for cholera (i.e. tetracycline) and for typhoid (i.e. chloramphenicol, trimethoprim-sulfamethoxazole or ampicillin) is now common in Asia and Africa.

In 1985, Multi Drug Resistant *E. coli* was isolated from water sources in Nyanza province, Kenya (Waiyaki *et al*., 1985). It was resistant to ampicillin, tetracycline, chloramphenicol, nalidixic acid and streptomycin. A study involving 325 multiple resistant Enterobacteriaceae isolates from patients attending hospital in Nairobi

between 1990-93 indicated high (>50%) resistance to amoxicillin, tetracycline, and trimethoprim/sulfamethoxazole (Kariuki *et al*., 1996). In 1997, MDR *S. typhi* emerged in Nairobi with increasing prevalence over time. In another study, all 64 isolates of *S. typhimurium* from Nairobi and the 40 isolates from Kilifi showed resistant to two or more drugs including cotrimoxazole, streptomycin, tetracycline and chloramphenicol (Kariuki *et al*., 2000). The use of amoxicillin and trimethoprim-sulfamethoxazole (SXT) as the drugs of choice for the treatment of drug susceptible typhoid fever was recommended after the emergence of resistance and a high relapse rate associated with chloramphenicol (Thisyakorn and Mansuwan, 1992). A study done by Brooks *et al*., (2006) in rural Western Kenya (1997-2003) showed that with the exception of *Campylobacter* spp., susceptibility to the antimicrobials used most widely in the community was low: less than 40% for all isolates tested and less than 25% for *Shigella* spp. Kariuki *et al*., (1994), isolated a high proportion of MDR *Shigella* spp to chloramphenicol, cotrimoxazole, streptomycin and tetracycline from HIV seropositive patients in Nairobi, Kenya. Another study done in Kenyatta National Hospital (Kenya) indicated that *Shigella* isolates were 100% resistant to SXT, tetracycline and streptomycin and 60% were resistant to ampicillin. Multidrug resistance was observed on all the bacteria studied (Ndung'u *et al*., 2004).

2.6 Importance of medicinal plants

2.6.1 Traditional medicine and Primary Health Care

Recently, there has been a worldwide increase in the use of traditional medicine. A big percentage of the world's population, particularly in the developing countries, depends almost entirely on plant derived medicines for treatment of most diseases. This is due to the unavailability and high cost of conventional drugs (Geoffrey, 1996). In India, for example, 70% of the population use traditional Indian medicine and about 80% of the African population depends on traditional medicine for their health care needs (Zhang, 2000). Traditional medicine forms part of the African culture and heritage. WHO has estimated their use in some African countries to be 90% Ethiopia, 70% Rwanda, 70% Benin, 69% Uganda, and Tanzania 60% (Murende, 2000).

More than 20% of the Kenyan population relies on traditional medicine as its primary source of healthcare, while more than 90% use medicinal plants at one time or another (Odera, 1997). This shows that traditional medicine continues to play a major role in primary health care services. They are more accessible than modern health facilities for most of the rural population in the country. They are also relatively inexpensive, locally available and usually accepted by the local communities as comparable to modern conventional medicine.

WHO, has consistently supported, promoted and assisted the development of traditional medicine in order to improve the African health agenda, particularly for the less-developed countries of the world, which are unable to provide for the population using modern health facilities (Liwen, 2003). This is through provision of guidelines for the development and utilization of indigenous system of medicine. The African heads of state declared the period 2001 – 2010 as a period for traditional medicine in Africa, tagged as the "Decade of traditional medicine in Africa" and the 31st of August every year was to be observed and celebrated as the African Traditional Medicine Day in all African countries (Elujoba *et al.*, 2005).

2.6.2 Role of medicinal plants in modern drug discovery and development

Medicinal plants contain substances known for their healing properties. The active principles differ from plant to plant but these active principles play an important role in conventional modern medicine since they may inhibit bacterial growth by different mechanisms than those presently used by conventional antimicrobials and may have a significant clinical value in treatment of resistant pathogens (Barbour *et al.*, 2004). The antimicrobial activity of medicinal plants occurs from the combination of secondary products present in the plant. The active compounds are used either directly or indirectly to treat diseases or

maintain health. Key classes of the active compounds include: Alkaloids, flavonoids, tannins, amino acids and oils. These and many other secondary compounds are the main focus of natural chemists, pharmacognosists and ethnopharmacologists (Martin, 2004) because they have diverse pharmacological effects.

Medicinal plants have contributed to drug discovery (Cragg and Newman, 2001). In 1785, the medicinal uses of *Digitalis purpurea* L. was discovered, which gave rise to digoxin, a cardiac drug. The analgesic morphine was isolated from Opium poppy flower (*Papaver somniferum* L.) for the first time by Friedrich Serturner in 1803 (Wildwood, 1998). Flouroquinone, an antimalaria compound was isolated from several medicinal plants during the same century (Huang *et al*., 1992). In the 1980's, taxol, which has an effect on HIV, was also discovered (Cox and Balick, 1994). This shows that the study of medicinal plants can provide lead compounds for new drugs for the pharmaceutical companies and therefore promote Primary Health Care in developing countries.

A study done in the United States from 1959 to 1980 indicated that 25% of prescriptions dispensed from community pharmacies contained plant extracts or active principles derived from higher plants and currently in use drugs. Presently, at least 119 chemical substances derived from 90 plant species can be considered important in one or more countries (Farnsworth *et al*., 1985; Cragg and Newman,

2001). In East Africa, for example, medicinal plants have been used as a source of lead compounds for the development of new antimalarial drugs (Waako *et al*., 2007).

2.6.3 Role of Ethnobotany in drug discovery

Ethnobotany as a multidisciplinary science that studies the interaction between plants and people helps in the documentation of the traditional knowledge of local communities (Flaster, 1996). According to Kone *et al*., (2004), it is possible to increase the chances of drug discovery by about four times when ethnobotanical survey is used and hence lead to validation of commonly used medicinal plants and in turn boost the confidence of users.

Many indigenous people are aware of some common plants that have medicinal uses (Balick and Cox, 1996). Kenya has a diverse and rich cultural mix of different ethnic groups, each of which has knowledge about medicinal plants that is passed orally from one generation to another (Njoroge, 2003). Currently there are about 119 clinically useful compounds derived from plants. There is a 74% correlation between the performance of these compounds and their use by traditional communities. This has given new direction to the use of medicinal plants (Coombes, 1992). Hence, ethnobotanical survey helps in identifying plants with high chances of possessing active compounds.

2.7 JUSTIFICATION

Diarrhoea disease is still one of the leading causes of mortality in developing countries, despite advances in understanding and management that have occurred over the years (WHO, 1996). They cause long-term infections due to resistance to antibiotics. There is therefore an urgent need for new types of medicines from medicinal plants that can be used together with antibiotics to enhance their activity against drug-resistant enteric bacteria.

Since it is known that some of the pharmaceutical products in the market today originated from traditional medicinal knowledge, and that ethnobotanical studies aid in selecting potential medicinal plants, the latter is therefore helpfull in identifying and selecting potential antidiarrhoeal medicinal plants. This will be very important in discovery of model molecules to be used in drug development.

There is continued loss of indigenous knowledge about medicinal plants due to death of holders of that knowledge, who are reluctant to share the information with other people. This study, through the ethnobotanical survey, will ensure that the knowledge is preserved.

Many pathogens can produce persistent diarrhoea and seriously affect growth, nutritional status and intellectual function. Some of these pathogens can have an

effect on children's growth even without causing diarrhoea. Hence there is need for antimicrobial studies in order to improve child health in the country.

It has been confirmed that water in the Lake Victoria region is contaminated with fecal matter (Boga *et al*., 2007) and is unlikely to be treated before it is used for domestic purposes. Therefore, this study focuses on enteric pathogens isolated from various stations along Lake Victoria. The emergence of antibiotic resistance among previously susceptible organisms poses a major public health problem and prevents successful therapeutic measures against diarrhoea in Kenya. Hence, the antibiotic sensitivity patterns of the test isolates need to be evaluated.

Toxicological activities of antidiarrhoeal medicinal plants also need to be assessed in order to determine their safety and hence, be able to avoid possible detrimental health risks due to toxic compounds that may be contained in the plants.

The results of this study will provide supportive information on the traditional usage of the studied plants and authenticate that some of the plant extracts possess compounds with antimicrobial properties that can be used as a basis for development of antimicrobial agents in new drugs for the therapy of diarrhoea due to enteric pathogens. This will pave way for further research to standardize their prescription and usage.

Medicinal plants are constantly diminishing due to over-exploitation and wrong harvesting methods. The results of this study will provide data necessary to promote the domestication and conservation of the antidiarrhoeal medicinal plants in order to make them more available to herbalists and other users.

2.8 HYPOTHESIS

Antidiarrhoeal medicinal plants used in traditional therapy have antimicrobial effect against enteric pathogens.

2.9 OBJECTIVES

General objective

To test the efficacy of antidiarrhoeal medicinal plants against clinical and environmental enteric pathogens

Specific objectives

1. To identify antidiarrhoeal medicinal plants commonly used by herbalists in Rachuonyo District.
2. To compare the antimicrobial activity of different extracts of the selected antidiarrhoeal medicinal plants against clinical and environmental enteric pathogens.

3. To evaluate for synergistic effects of selected antibiotics and the active plant extracts on antibiotic-resistant enteric pathogens.
4. To screen for the presence of phytochemicals in the most commonly used antidiarrhoeal plants.
5. To evaluate the selected antidiarrhoeal medicinal plants for toxicity.

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 Sampling and selection of plants

Plant materials were collected from Rachuonyo District in Nyanza Province, around Lake Victoria. Rachuonyo lies within longitudes 33º20′E and 35º20′E and latitudes 0º20′S and 0º50′S (Figure 1). It covers an area of 925 km^2. The district is the fifth most densely populated in Kenya, with a population of 307,126. It has an extremely high disease burden (CBS, 1999). An outbreak of cholera was first reported on 27th August 1997 in the district, after which, the other 5 districts of Nyanza province were affected. The district is constantly affected by diarrhoea, especially due to cholera, because of the frequent problem of flooding (IFRC, 1997).

An ethnobotanical survey was conducted in order to identify the plant species mostly used in the treatment of diarrhoea diseases in the district. This involved interviews with herbalists and clients of the herbalists using semi-structured questionnaires, group discussions and observations (Cunningham, 2000). Four divisions were selected, from which 5 villages were randomly selected. At least 10 households were randomly selected from each village and interviewed. The questionnaires were distributed after prior informed consent was sought from the respondents.

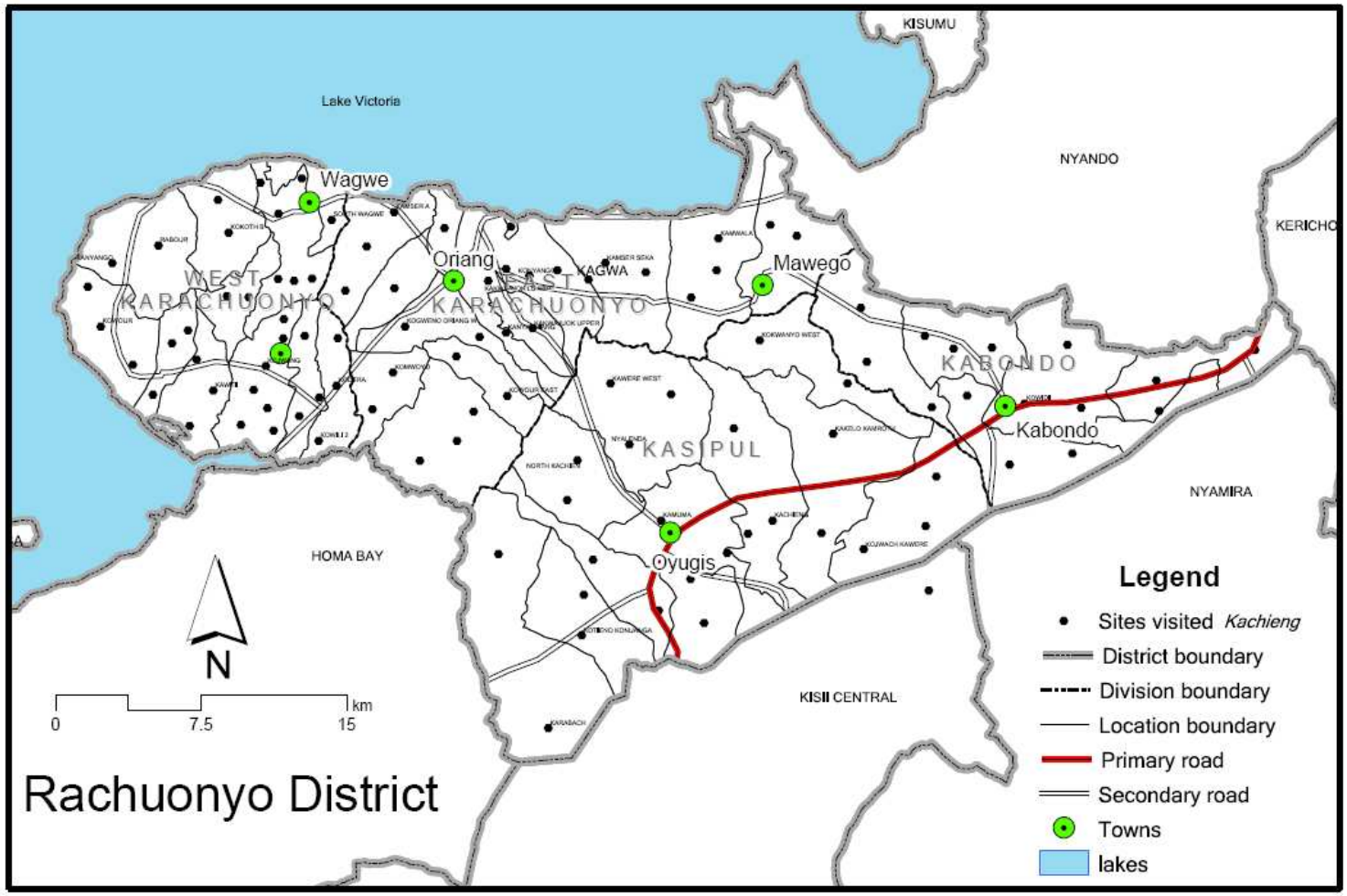

Figure 1: Map of Rachuonyo district showing field sites visited

The information collected included the local names of the plants, the parts of plants that are used, mode of preparation, the mode of administration, and the curative properties of the plants. Any language barrier was avoided by having an interpreter. The criterion used for selecting the plant species and plant parts was based on the community's medicinal uses, which increases the chances of drug discovery (Kone *et al*., 2004). The fidelity level (FL) method was used in the selection of plants for further studies. The FL is the percentage of informants claiming the use of a certain plant for the same major purpose (Teklehaymanot and Giday, 2007). It was calculated for diarrhoeal diseases as:

$$FL(\%) = \frac{Np}{N} \times 100$$

Where:

Np=Number of informants that claim a use of a plant species to treat a particular disease.

N=Number of informants that use the plants as medicine to treat any given disease.

The plants were collected from the villages by conducting return-visits and transect walks with a key informant. They were identified and preserved at the Jomo Kenyatta University of Agriculture and Technology (JKUAT) Botany department herbarium. The reference numbers of the specimens were also recorded. Identification was done up to genus and species of the plants by a

qualified botanist (Martin, 2004). Preservation was done by placing the plant specimens between two sheets of newspapers and placing them on a drying presser. The sheets of newspapers were changed daily to prevent growth of moulds from accumulated moisture. These were done for approximately 3 weeks or until they were completely dry (Martin, 2004).

3.2 Extraction of selected medicinal plants

The plant parts were dried at ambient temperature under shade after collection until they were dry. For laboratory analysis, they were ground into powder form using a grinder (MK 10-525-B). Sequential soxhlet extraction of active compounds in the medicinal plants was done using solvents of increasing polarity (hexane, acetone and methanol). The powdered plant material (50 g) was soaked overnight in 95 % hexane and then soxhlet extracted for 10 h until the entire coloured compound had been removed and the solvent is clear. The residue was then soaked overnight in 95 % acetone and then soxhlet extracted for 10 h until the entire coloured compound had been removed and the solvent was clear. The residue was then soaked overnight in 95 % methanol and then soxhlet extracted for another 10 h. The extracts obtained in each step were concentrated under vacuum at 40°C by using a rotary evaporator to remove the excess solvents. The crude extracts were further dried by evaporation by placing the beaker containing the extract on a boiling water bath until the extracts were completely dry. The plant extracts that were not to be used immediately were stored at 4°C

until use (Kone *et al*., 2004). Standard solutions of the extracts were prepared using the solvents that were used for the extraction. Hot water extraction was employed to simulate the traditional method used for preparing herbal medicine. The powdered plant material (50 g) was weighed and soaked in 1 litre of water in a flask and then boiled for 4 hours under slow heat, while swirling the flask. The mixtures were then allowed to stand for 30 minutes. The extracts were then filtered with a Whatman's number 1 filter paper. Each infusion was always freshly prepared just before use.

3.3 Sampling of environmental water and isolation of enteric pathogens

Isolates of enteric pathogens from water samples were collected aseptically from various environmental sources around the Lake Victoria region in a previous study by Mutuku (2006). The isolates were stored at 4^{o}C at the Centre for Microbiology Research, KEMRI, Nairobi, until they were used in this study.

3.4 Purification and storage of bacterial isolates

The identified organisms were purified by plating on MHA plates followed by incubation at 35°C for 24 h. After inoculation, a loopful of each organism was emulsified in 1 ml stocking vials containing Trypticase Soy broth with 15 % glycerol from where they were sub-cultured for the sensitivity tests. The vials were stored at -70°C.

3.5 Verification of Enteric pathogens

3.5.1 Selection of colonies

Verification of the enteric pathogens was done by primary identification using colony colour, morphology and gram staining. MacConkey medium is selective for lactose fermenters, which appeared as red/pink colonies. On Eosin Methlyene Blue (EMB) medium, lactose fermenters appeared as green-black or metallic colonies. Growth of non-lactose fermenters was inhibited by methylene blue. Xylose Lactose Deficient (XLD) medium is a selective differential medium for isolation of *Salmonella* and *Shigella* spp. Pink/red colonies with black centers represented *Salmonella* and those without black centers represented *Shigella*. *Salmonella*, *Shigella* (SS) medium is a selective medium for isolation of *Salmonella* and *Shigella* spp. The colonies that were selected were either pink or pale with or without black centers. *Vibrio cholerae* was isolated by sub-culturing on Thio Citrate Bile Salt (TCBS) agar. Oxidase tests were done on the non-lactose fermenters to distinguish *Pseudomonas aeruginosa* from other enteropathogens (Bergy *et al*., 2001; Cheesbrough, 2000; Cappuccino, 2002).

3.5.2 Biotyping with test-tube media

Biochemical tests were done to further identify the organisms. This was done using Triple Sugar Iron (TSI) medium (Oxoid, UK), Lysine Indole Motility (LIM)

medium (Nissui, Japan), Simmon's Citrate (SC) medium (Oxoid, UK), Methyl Red-Voges Proskauer (MR-VP) medium (Oxoid, UK) and Urea medium (Nissui, Japan). The identification was performed as described in Bergy's manual of determinative bacteriology (Bergy *et al*., 2001). The procedure is shown in Appendix 2. The results were interpreted as described in appendix 3 (Bergy *et al*., 2001; Cappuccino, 2002).

3.5.3 Analytical Profiling Index (API 20E)

The organisms were further identified using the API-20E test system. The system has 20 microtubules containing dehydrated substrates. The organisms were inoculated in Mueller-Hinton agar plates and incubated overnight at 35˚C. After incubation, 1-2 colonies were picked from the MHA plates and emulsified in sterile normal saline to match a McFarland 0.5 standard tube. The API-20E cupules were inoculated as instructed by the manufacturer (API system, France). The strips were incubated for 24h at 35°C. During incubation, the metabolites produced colour changes that are spontaneous or are revealed after the addition of reagents. The results were read with the aid of a profile recognition system (API 20E Analytical Profile Index).

3.6 Environmental and Clinical isolates used in Bioassays

The biological assays were carried out on 14 enteric pathogens isolated from various wetlands found around the shores of Lake Victoria region, 2 reference cultures and 5 clinical isolates of enteric pathogens obtained from KEMRI-CMR. Strains of *Staphylococcus aureus* were included in the assays to represent the gram-positive microorganisms, which helped to determine whether the medicinal plants under study have broad-spectrum activity. The various isolates under study are shown in Table 1 below.

Table 1: Bacteria strains used for Bioassays

Microorganisms	**Source**	**Type/Isolate**
Staphylococcus aureus	KEMRI-CMR	ATCC 25923
Escherichia coli	KEMRI-CMR	ATCC 25922
S. aureus	KEMRI-CMR	Clinical
E. coli	KEMRI-CMR	Clinical (01:H7)
Shigella dysenteriae	KEMRI-CMR	Clinical
Salmonella typhi	KEMRI-CMR	Clinical
Vibrio cholerae	KEMRI-CMR	Clinical
E. aerogenes (Isolate 2)	River Yala	Environmental
Klebsiella spp. (Isolate 9)	River Nzoia	Environmental
Shigella flexneri (Isolate 12)	Chemelil effluent	Environmental
S. aureus (Isolate 6)	River Mbogo	Environmental
Proteus mirabilis (Isolate 10)	River Nyando	Environmental
Proteus spp. (Isolates 7 and 15)	River Mbogo, Chemelil effluent	Environmental
P. aeruginosa (Isolates 11 and 13)	River Kisat, Chemelil effluent	Environmental
E. coli (Isolates 3, 4, 5, 8 and 14)	Rivers Kisat, Nyando and Mbogo and Chemelil effluent	Environmental

3.7 Antimicrobial assays

3.7.1 Antibiotic susceptibility tests

Susceptibility of the test organisms to commercial antibiotics was determined using the disc diffusion methods adopted from the Kirby-Bauer technique (Bauer *et al*., 1966). After isolation of the enteric pathogens, an inoculum was prepared by emulsifying 3 colonies from the starting culture in 2 ml Mueller-Hinton Broth (MHB) and incubating overnight at 35°C. Then 0.5 McFarland turbidity was prepared for each isolate in sterile 0.9 % normal saline. (MHB) was used as the diluent to make the turbidity adjustment of the growth to match Barium chloride suspension turbidity equivalent to McFarland 0.5, using a spectrophotometer. An aliquot (100 µl) of the inoculum was spread-plated out onto Mueller-Hinton agar (MHA) plates using a sterile cotton swab. The antibiotic disks (6 mm in diameter) obtained from Becton Dickinson and Co., Cockeysville, MD 21030 (Appendix 6), were then placed aseptically on the seeded plates. *Escherichia coli* ATCC 25922 was used as a control for growth and disk potency. The plates were incubated aerobically for 24-48 h at 35°C, after which they were examined for zones of inhibition. The inhibitory diameters were measured using a ruler. The zones of inhibition were compared with a chart showing standard inhibitory diameters of the antibiotics. The procedure was repeated twice for all the test organisms. The data was entered in Excel spreadsheets and used to construct graphs. The susceptibility results were interpreted as described by the National Committee for

Clinical Laboratory Standards (NCCLS, 2002). The antibiotic discs that were used for the test and their concentrations are shown in appendix 6.

3.7.2 Antimicrobial assay of the plant extracts

Susceptibility of the test organisms to crude extracts was also done according to Kirby-Bauer technique (Bauer *et al*., 1966). The bacterial cultures were grown on MHA overnight at 35ºC. The extracts (0.1 g) were weighed and mixed with 1 ml of DMSO, to make a concentration of 100 mg/ml for each medicinal plant. After preparation of the inoculum, sterile swabs were used to spread plate the inoculum onto MHA plates. Using a paper punch (6 mm), filter paper discs were prepared. They were sterilized by autoclaving at 121ºC for 15 min. in a well-sealed universal bottle (Ogao et al., 2004). Subsequently, the sterile filter papers discs (6 mm in diameter), inoculated with 10 µl of the extracts, were placed on the surface of each inoculated plate using sterile forceps. The plates were allowed to stand for about 30 minutes before incubation at 35^{o}C for 18-24 h. The controls were discs soaked with the solvents that were used to prepare the extracts. They were prepared by inoculating 10 µl of each of the solvents used to prepare the plant extracts onto sterile filter paper discs and allowing them to dry. The plates were incubated overnight at 35ºC for 24-48 h, after which they were examined for zones of inhibition. The inhibitory diameters were measured using a ruler. The

procedure was done in duplicates. The extracts showing some inhibitory effect were used to determine the Minimum Inhibitory Concentration for each bacterial sample (Cheesbrough, 2000). The data was entered in Excel spreadsheets and analyzed using SAS version 8 statistical software for the analysis of variance. The means were separated using Student-Newman-Keuls test.

3.7.3 Determination of Minimal Inhibitory Concentration

The Minimum Inhibitory Concentration (MIC) of the plant extracts to the bacterial isolates was determined according to Cheesbrough (2000). The inoculum was prepared as previously described. Doubling dilutions of the plant extracts were done from 10^{-0} to 10^{-6} by adding 100 µl of neat extract (100 mg/ml) into 100 µl of sterile distilled water in a well in a microtitre plate. Aliquots (100 µl) were introduced to each of the appropriately labeled wells in the microtitre plate serially, beginning from the first well up to the last dilution. A well containing sterile distilled water without added plant extract was included in each assay. Then 10 µl was taken from each dilution and inoculated onto paper discs (6 mm). They were allowed to air-dry and then placed aseptically onto seeded MHA plates and pressed lightly to hold them in place. The plates were incubated at 35˚C for 18-24 h. Then the zones of inhibition were observed and the lowest concentration to produce a zone of inhibition was considered the MIC.

Using a wire-loop to touch some parts of the zone of inhibition and streaking it out onto Mueller-Hinton agar plates was done to subculture the last dilution not showing visible growth. This was done to determine the Minimum Bactericidal Concentration (MBC) for each plant extract against the reference cultures of *S. aureus* and *E. coli*, which is interpreted as the lowest concentration of the plant extracts required to kill bacteria.

3.7.4 Synergistic interaction of plant extracts with antibiotics on resistant bacterial strains

In the present study, the synergism tests were evaluated using the disc diffusion assay (Ahmad and Aqil, 2006). Mueller-Hinton agar plates were inoculated with a suspension of the test isolate adjusted to a 0.5 McFarland standard according to the standard NCCLS disc diffusion susceptibility testing methodology (NCCLS, 2002). Sterile paper discs (6mm in diameter) were inoculated with 10 µl of the appropriate concentration of each of the active plant extracts and allowed to air dry. Then the antibiotic discs to which the bacteria were resistant to were placed on the plates. A disc containing the extract was placed at a pre-determined distance (6 mm apart) from the antibiotic discs. The plates were incubated overnight at 35ºC, after which the zones of inhibition were measured. These were compared to the zones of inhibition of the individual antibiotic discs. The

measurements were used to determine whether there was any synergism between the extracts and the antibiotics. The formation of a characteristic augmentation of the antibiotic disc inhibition zone adjacent to the plant extract disc was indicative of synergy between the antibiotic discs and the discs containing the plant extracts, at appropriate concentrations. Four antibiotics to which the test isolates were resistant were used for the evaluation of synergism. These were Ampicillin (10 µg/ml), Gentamicin (10 µg/ml), Cefuroxime (30 µg/ml) and Cotrimoxazole (23.75/1.25 µg/ml). Even though the MICs for 14 bacterial strains were determined, only 6 of them were considered for the synergism test, each representing a different group of organism (Ahmad and Aqil, 2006)

3.7.5 Determination of the kill kinetics of the plant extracts

The time-kill kinetics of antimicrobial activity is generally used to evaluate and compare new drugs and to study differences in antimicrobial susceptibility of clinically important bacterial isolates (Amsterdam, 1991).

The bacteria were grown on Mueller-Hinton Agar (MHA) and incubated at 35^{o}C for 24 h to get discreet colonies. Log phase cultures were obtained by suspending about 10^{6} to 10^{7} CFU/ml (1-2 colonies) in Mueller-Hinton broth and incubating them at 35^{o}C for 24 h. The turbidity was monitored using a spectrophotometer at

6h intervals, in order to obtain the growth curve of the organisms. This was done at intervals of 6 hours. Thus, the exponential stage of the organisms was known. This was done in duplicates. Then 1 ml of 100mg/ml bioactive extract was dissolved in 9 ml of sterile MHB to make 10 ml, then doubling dilutions were made in normal saline to form concentrations of 100, 50, 25, 12.5 and 6.25 mg/ml. Log phase cultures of the organisms (20 µl) were added to 10 ml of the dilution corresponding to the MBC of the extract and incubated overnight at 35^{o}C. Initial control counts were obtained by doing serial dilution and spread plating 0.1 ml of the inoculum on MHA just before incubation. Subsequently, 0.1 ml of sample from each extract concentration was serially diluted and spread plated at intervals of 6 h, 12 h and 24 h. All spread plates were allowed to stand for 30 minutes at 4^{o}C before incubation to allow the antimicrobial elements to soak and release the viable microbial cells (Okemo *et al*., 2004). The plates were then incubated at 35^{o}C for 24 h. Those plates containing between 30-300 colonies for each series of dilutions were counted and the means of the readings were calculated and recorded in Excel spreadsheets. They were then used to construct a growth profile for the organisms (Amsterdam, 1991; Okemo *et al*., 2004).

3.8 Phytochemical analysis of the plant extracts

Preliminary phytochemical analysis of the crude extracts of the plants was performed by standard colour tests according to Chhabra *et al*., (1984) and Harbone, (1973). Various colour changes were observed to determine the

presence of possible groups of phytochemicals (Appendix 4). Thin Layer Chromatography (TLC) was used to confirm the results of the standard colour tests. Glass plates pre-coated with Silica gel Kieselgel DGF254 (Kobian Ltd., Kenya) were activated in the oven at 105ºC before loading an aliquot of each extract as a spot. These plates were then developed with Dichloromethane: Methanol (98:2) with five drops of glacial acetic acid and Hexane: Methanol (55:45) which eluted components into streaks and spots appropriately. The components were visualized under visible and ultra violet light (254 and 366 nm) and sprayed with specific visualization (chromogenic) reagents (Appendix 5) in order to reveal different groups of chemical constituents.

3.9 Toxicity screening

The brine shrimp lethality test (BST) was done to detect the presence of cytotoxic activity in the plant extracts. Artificial seawater was prepared by dissolving 33 g of sea salt in one litre of distilled water. Brine shrimp (*Artemia salina*) eggs were incubated in 80 ml artificial seawater (ASW). Dilutions of the plant extracts were made in Dimethyl sulfoxide (DMSO) in triplicate test tubes. After hatching, 10 brine shrimp larvae (nauplii) were placed in each of the test tubes containing different concentrations of the plant extracts. Control brine shrimp larvae were placed in a tube containing ASW and DMSO only. The initial numbers of Brine shrimps in each tube were counted. After 24 h, the numbers of live nauplii were recorded for each concentration. This allowed the calculation of the cytotoxicity

measure called LD_{50} value (lethal dose capable of killing 50% of the organisms) of each extract. Cytotoxic activities were considered significant if the LD_{50} values of less than 30 µg/ml were observed (Saupe, 2005). Plant extracts with LD_{50} values greater than 100 µg/ml were considered non-toxic and hence safe for treatment in human beings (Mbwambo *et al*., 2007; McLaughlin *et al*., 1991). This indicates that values between 30 µg/ml and 100 µg/ml are considered a safe dose.

CHAPTER 4

4.0 RESULTS

4.1 Ethnobotanical survey

During the ethnobotanical survey, 191 respondents were interviewed (147 female and 44 male) (Appendix 2). The age bracket of the respondents was 18 years to 76 years (mean age=52), with most of them having primary school level of education. The main source of income in the area is peasant farming. Only one herbalist (male) was willing to be interviewed.

The respondents identified 20 plant species as the ones regularly utilized in the management of diarrhoea (table 2). Some of the frequently used plant species included: *Aloe secundiflora* Engl., *Euclea divinorum* Hiern, *Lannea schimperi* (Hochst.) Engl, *Lippia javanica* (Burm.f.) Spreng., *Psidium guajava* L., *Albizia coriaria* Welw. Ex Oliv., *Melia volkensii* Gurke and *Terminalia brownii* Fres. Of these, 4 were chosen because they had the highest FL values (percentage of informants claiming the use of a certain plant for the same major purpose) and they were the most frequently mentioned as the ones used for the treatment and management of diarrhoea. They are used either alone or in combination with other medicinal plants in the treatment of diarrhoea. The medicinal plants that are used as remedies of a single ailment have 100% fidelity level than those that are used as remedies for more than one type of ailment. For example, *Melia volkensii* Guerke is used to treat diarrhoea, stomachache, measles and other skin infections

among other ailments and its FL value is 27%. On the other hand, *Zanthoxylum gilletii* De wild is only used to treat diarrhoea and its FL value is 100%. In this study, *Melia volkensii* Guerke was chosen over *Zanthoxylum gilletii* De wild because it was the most frequently mentioned among the two. Some of the plants do not have local Luo names. For instance, *Melia volkensii* Guerke and *Psidium guajava* L. use Swahili names (Mwarubaini and mapera respectively). This is because they are not indigenous in the area.

From the survey, it was observed that some of the plants used to treat diarrhoea are not easily available because they are decreasing with time. This may be due to long spells of dry season experienced in the country. Their availability varies with weather/season. They are more available during the rainy season than during the dry season.

Table 2: Ethnobotanical information on the plant species commonly used in management of diarrhoea in Rachuonyo district

Plant species (Family)	Luo name	Part used	Method of preparation	F	FL (%)
Terminalia brownii Fres (Combretaceae)	Onera	Stem bark	Boiling	23	100
Albizia coriaria Welw. Ex Oliv. (Leguminosae-Mimosodieae)	Ober	Stem bark	Boiling	22	100
Aloe secundiflora Engl. (Aloaceae)	Okaka	Leaves	Boiling	17	100
Zanthoxylum gilletii De wild (Rutaceae)	Sogo	Stem bark	Boiling	2	100
Lannea schimperi Engl. (Anacardiaceae)	Kuogo	Stem bark	Boiling	5	71
Hydnora abyssinica Schweinf (Hydnoraceae)	Oyuso	Roots	Boiling	1	50
Grewia mollis Juss (Tiliaceae)	Powo	Leaves/stem bark	Boiling	2	40
Euclea divinorum Hiern (Ebenaceae)	Ochol	Leaves/Root	Crushed in warm water	6	38
Psidium guajava L. (Myrtaceae)	Mapera	Leaves	Boiling or crushed in warm water	2	33
Ekebergia capensis Sparrm (Meliaceae)	Tido	Root bark/leaves	Crushed and mixed with warm water	1	33
Melia volkensii Guerke. (Meliaceae)	Mwarubaini	Stem bark/leaves	Boiling	21	27
Rhus vulgaris Meikle (Anacardiaceae)	Awayo	Leaves	Crushed in hot water	1	25
Annona senegalensis Pers. (Annonaceae)	Obolo	Roots	Boiling	1	20
Solanum icanum L (Solanaceae)	Ochok	Roots	Boiling	1	14
Plectranthus barbatus Andrew (Labiataceae)	Okita	Leaves	Crushed and soaked in warm water	1	13
Sapium ellipticum Pax. (Euphorbiaceae)	Achak	Root bark/Leaves	Boiling	1	11
Schkuhria pinnata Thell. (Compositae)	Onyalo biro	Leaves	Boiling	1	11
Lippia javanica Spreng (Verbanaceae)	Omieny	Roots and leaves	Boiling	4	9
Ageratum conyzoides L. (Asteraceae)	Oluoro chieng	Whole plant	Boiling	1	6
Tithonia diversifolia (Hemsl.) A. (Asteraceae)	Akech	Whole plant	Boiling	2	4

NB: F=Frequency; FL=Fidelity level

4.2 Antimicrobial assays

4.2.1 Antibiotic Susceptibility tests

The bacteria that were used to carry out the antibacterial susceptibility tests included 5 clinical isolates obtained from KEMRI-CMR and 14 environmental enteric pathogens that were isolated from water samples collected from rivers and effluents around the Lake Victoria region (table 1). The highest resistance was to Ampicillin (10 µg/ml) followed by Cefuroxime (30 µg/ml) (Figure 1). Of the clinical isolates, only 2 (25 %) were resistant to Ampicillin. These were *Vibrio cholerae* and *Shigella dysenteriae*. The rest (5) were sensitive to all the antibiotics tested (Table 3). Among the environmental isolates, 42.9 % (6) were resistant to one or more antibiotics, while 57.1 % (8) were sensitive to all the antibiotics tested. Of the resistant isolates, two (2) were resistant to Ampicillin, Cefuroxime, Cotrimoxazole (23.75/1.25 µg/ml) and Gentamicin (10 µg/ml). Two isolates, *E. aerogenes* (isolate 2) and *Klebsiella spp* (isolate 9) were resistant to Cefuroxime alone. *Pr. mirabilis* (isolate 10) was resistant to Ampicillin, Gentamicin, and Cefuroxime. *E. coli* (isolate 14) was resistant to Ampicillin, Cotrimoxazole and Cefuroxime (Plate 1). *P. aeruginosa* (isolate 11) was resistant to Ampicillin and Cefuroxime while *P. aeruginosa* (isolate 13) was resistant to Ampicillin alone, with intermediate resistance to Cefuroxime. The resistance patterns of the test isolates are shown in figure 2.

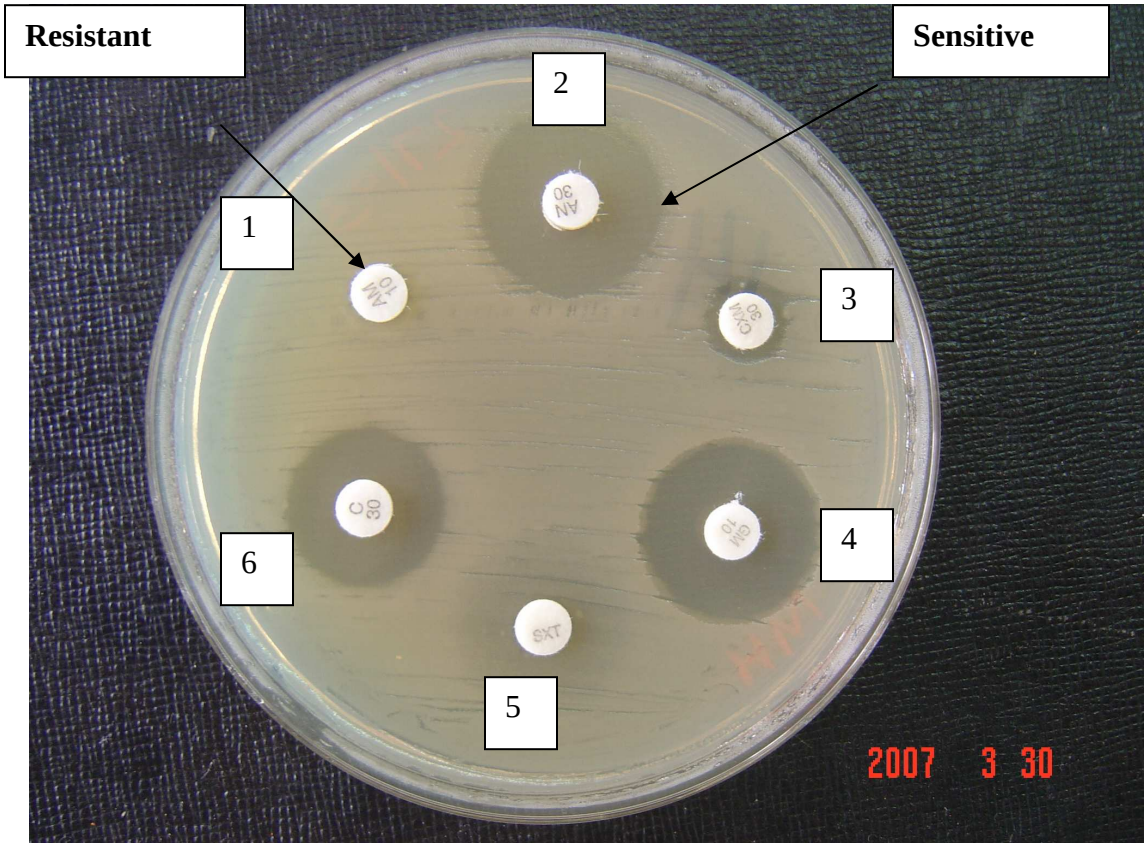

Plate 1: Antibiotic resistance pattern for *E. coli* (isolate 14) to (1) Ampicillin; (2) Amikacin; (3) Cefuroxime; (4) Gentamicin; (5) Cotrimoxazole and (6) Chloramphenicol

Table 3: Antibiotic resistance patterns of Clinical enteric pathogens

Antibiotic	**Sensitive (S)**	**Intermediate (I)**	**Resistant (R)**	**% Resistant**
Amikacin	5	0	0	0
Ampicillin	5	0	2	28.6
Chloramphenicol	5	0	0	0
Cotrimoxazole	5	0	0	0
Gentamicin	5	0	0	0
Cefuroxime	5	2	0	0

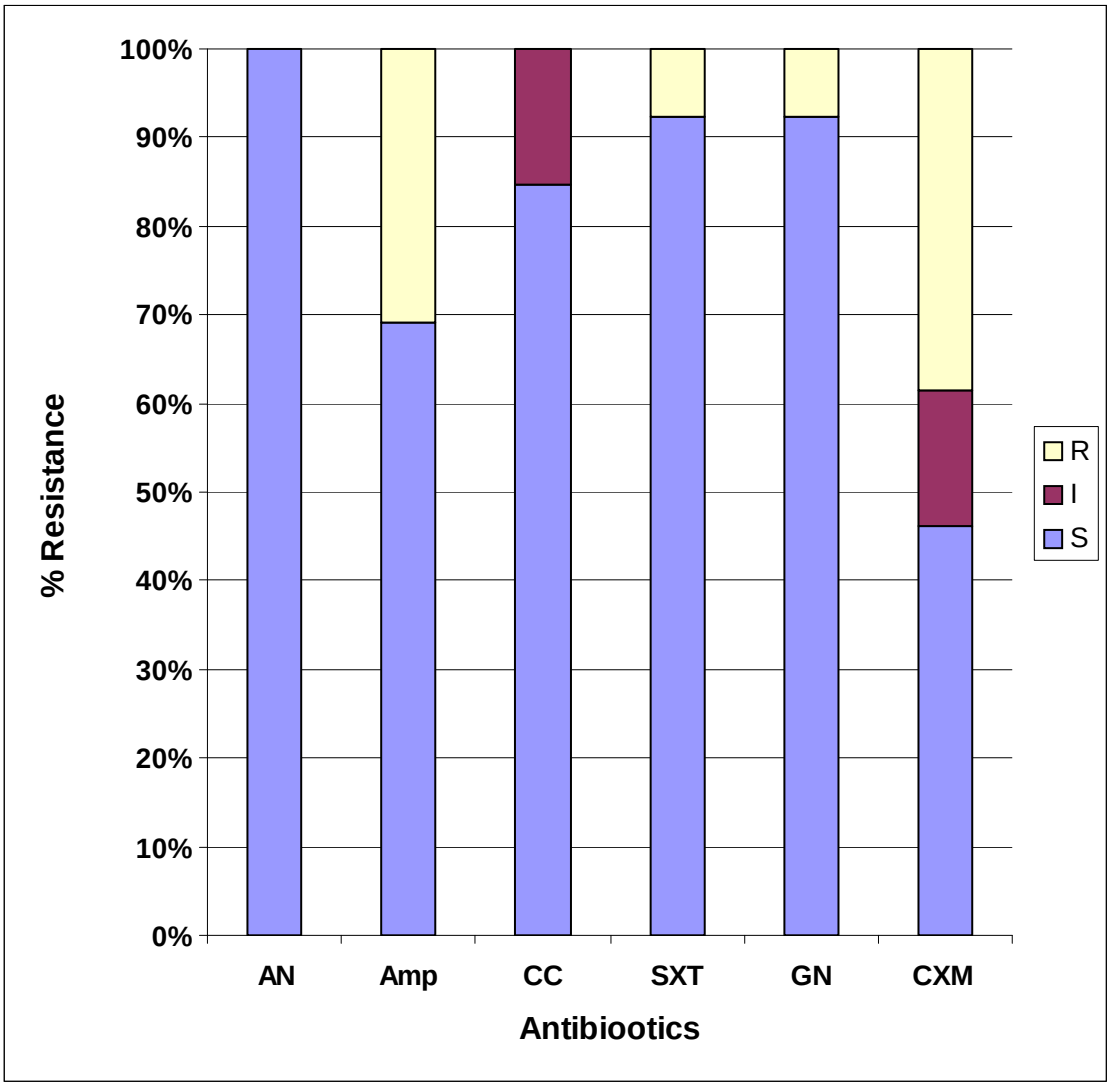

Figure 2: Antibiotic resistance patterns of 14 environmental enteric pathogens to AN- Amikacin, AM- Ampicillin, CC- Chloramphenicol, CXM- Cefuroxime, GM- Gentamicin, and SXT- Cotrimoxazole

Legend: R=Resistant; I=Intermediate Resistance; S=Sensitive

4.2.2 Antimicrobial assay of plant extracts

From the antimicrobial bioassay of the plants, varying levels of antibacterial activity were observed in most solvent fractions with zones of inhibition ranging from 7 mm for methanol extracts of *Melia volkensii* bark against *Shigella dysenteriae* (clinical isolate) to 24 mm for methanol extracts of *Terminalia brownii* bark against *Vibrio cholerae* (clinical isolate) (Appendix 7). Zone sizes of 6 mm (equivalent to the size of the discs) indicated no activity while zone sizes equal to or greater than 7 mm indicated activity. The controls were the solvents used for the extraction and they showed no inhibitions, hence any inhibitions observed in the plant extracts were not due to the solvents. Water, methanol, acetone and hexane extracts from *Aloe secundiflora* had very low activity against the clinical and environmental test isolates.

A high activity was shown by methanol, hexane and water extracts of *Terminalia brownii* (Figure 2). However, acetone extracts were inactive on all microorganisms (Appendix 7). Of the 21 test isolates, 9 (42.9 %) were sensitive to hexane extracts. Of these, 3 (33.3 %) were clinical isolates while 6 (66.7 %) were environmental isolates. Methanol extracts had activity on 7 (33.3 %) of the test isolates, of which 3 (42.9 %) were clinical isolates while 4 (57.1 %) were environmental isolates, one of which was *Proteus spp* (isolate 15). Water extracts

had the highest activity by being active against 12 (52.4 %) of the test isolates, of which 6 (50 %) were clinical isolates and 6 (50 %) were environmental isolates.

Among the extracts from the bark of *Melia volkensii*, acetone extracts had the highest activity against 9 (42.9 %) of the test isolates. Of these 4 (44.4 %) were clinical isolates while 5 (55.6 %) were environmental isolates. Water extracts showed slight activity on 4 (19.1 %) of the test isolates, none of which were clinical isolates. The rest of the extracts (hexane and methanol) from the bark of *Melia volkensii* had no activity on any of the test isolates.

Among the extracts from the leaves of *Melia volkensii*, the hexane and methanol extracts had no activity at all, but acetone and water extracts showed slight activity against environmental isolates (Appendix 7). Acetone extracts were active on *Enterobacter aerogenes* (environmental isolate). Water extracts inhibited 4 (19.1 %) of the environmental isolates.

Hexane and acetone extracts from *Albizia coriaria* were inactive on all test microorganisms. However, methanol and water extracts showed some activity on several microorganisms, such as *Proteus spp* (isolate 15) as shown on plate 2. Methanol extracts were active against 8 (38.1 %) of the test isolates, of which 2 (25 %) were clinical and 6 (75 %) were environmental isolates. The water extracts

were active against 5 (23.8 %) of the isolates, none of which were clinical isolates.

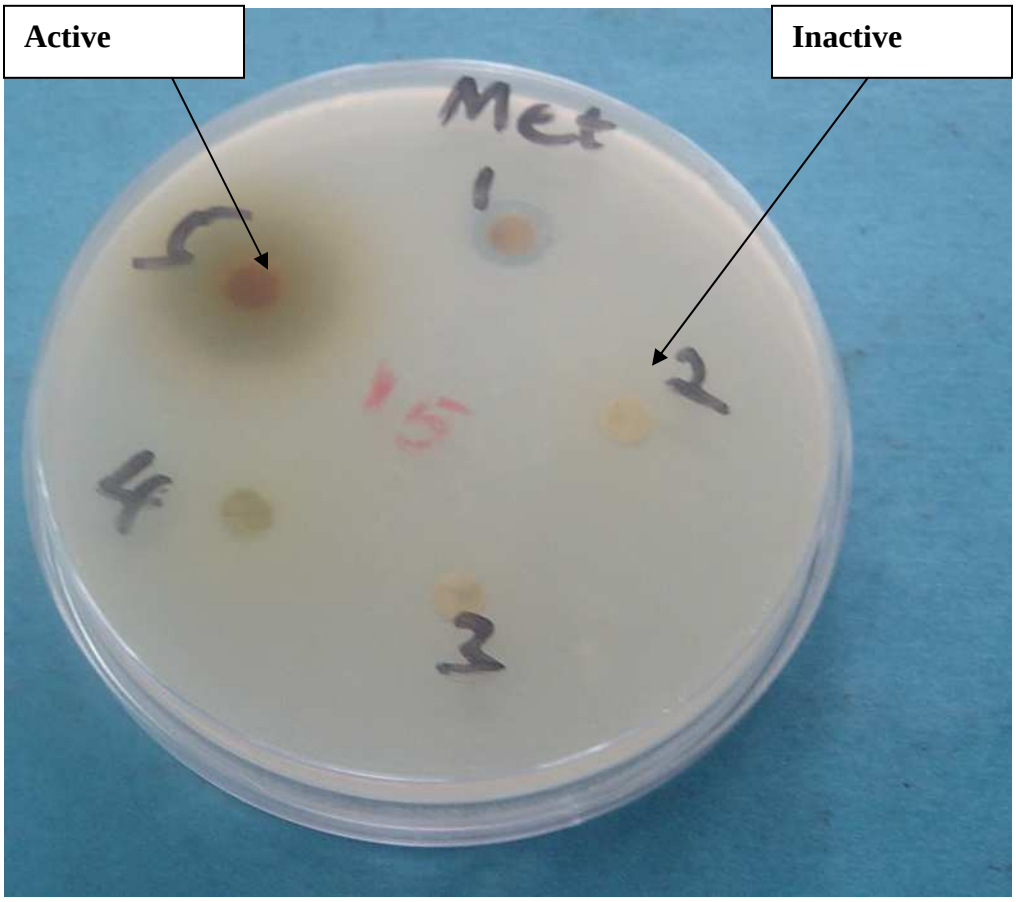

Plate 2: Antimicrobial assay for methanol extracts of (1) *Albizia coriaria* bark (2) *Aloe secundiflora* leaves (3) *Melia volkensii* bark (4) *Melia volkensii* leaves and (5) *Terminalia brownii* bark against *Proteus* spp (isolate 15)

Acetone extracts were significantly different from the other extracts at $p<0.0001$, with *Melia volkensii* (bark) having the highest mean activity of 10.95 mm. This

shows that *Melia volkensii* (bark) acetone extracts were significantly different from the other acetone extracts. Hexane extracts were also significantly different from each other in their activity at $p<0.0001$, with *Terminalia brownii* (bark) having the highest mean activity of 9.38 mm. Methanol extracts were also significantly different from each other at $p=0.0004$, having *Terminalia brownii* (bark) with the highest mean activity of 7.14 mm. Water extracts were significantly different from each other at $p<0.0001$, having *Terminalia brownii* (bark) with the highest mean activity of 8.86 mm.

Therefore, among the extracts, acetone extracts produced the highest mean activity against the enteric pathogens, followed by methanol, hexane and water respectively. *Terminalia brownii* produced the highest mean activities, followed by *Melia volkensii*, then *Albizia coriaria* and lastly by *Aloe secundiflora*. The mean activities are shown in figure 3.

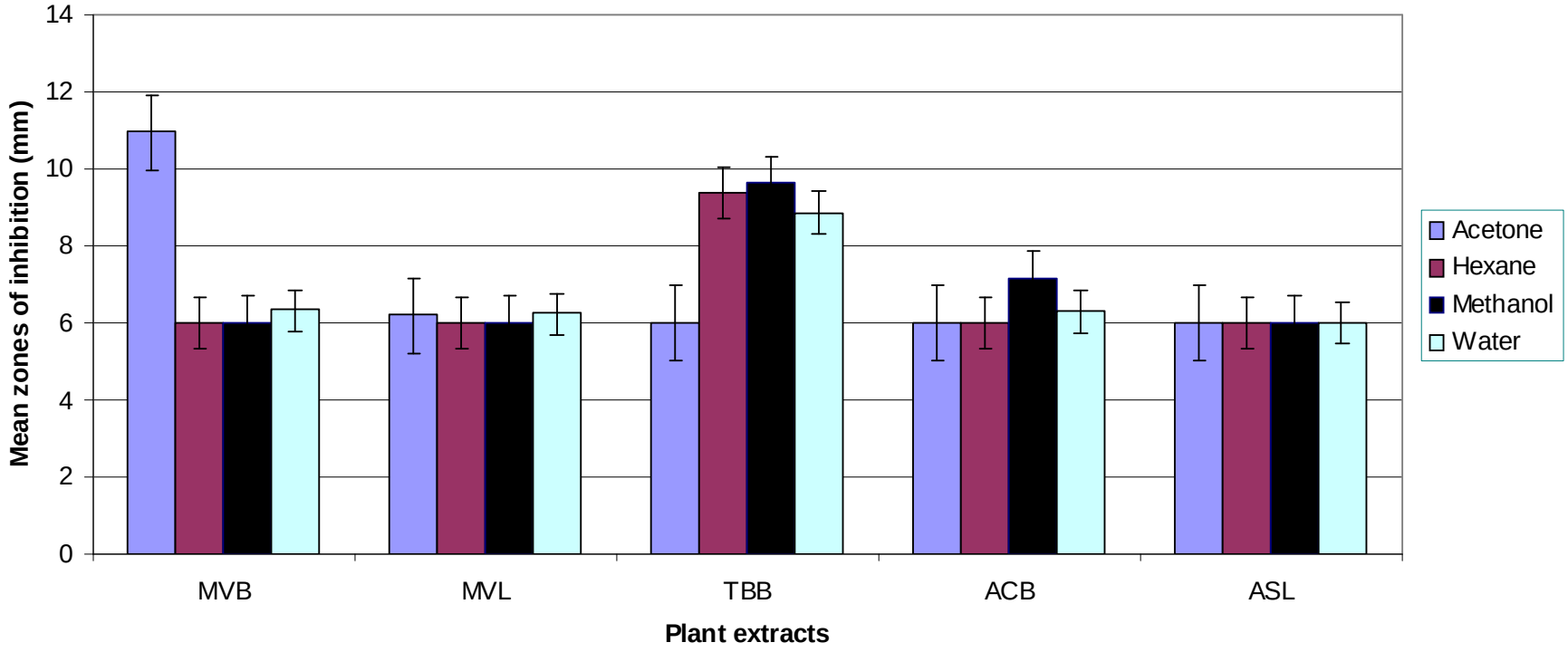

Figure 3: Mean activity of extracts of MVB: *Melia volkensii* bark; MVL: *Melia volkensii* leaves; TBB: *Terminalia brownii* bark; ACB: *Albizia coriaria* bark and ASL: *Aloe secundiflora* leaves against enteric pathogens

4.2.3 Determination of the Minimum Inhibitory Concentration

The MIC of the plant extracts ranged between 3.13 mg/ml for *Melia volkensii* against *Shigella dysenteriae* to 50 mg/ml for *Albizia coriaria* against *E. coli* ATCC 25922. The most active extract was *Terminalia brownii*, which was inhibitory on *Shigella dysenteriae* (plate 3) and *Staphylococcus aureus* at 3.13 mg/ml. The MIC varied within a particular microorganism. For instance, the MIC for *Shigella flexneri* (isolate 12) varied from one extract to another [hexane extract of *Terminalia brownii* bark (12.5 mg/ml), acetone extract of *Melia volkensii* bark (12.5 mg/ml), methanol extract of *Albizia coriaria* bark (25 mg/ml) and methanol extract of *Terminalia brownii* bark (6.25 mg/ml)]. The MIC also varied within a particular medicinal plant. For instance, for hexane extracts of *Terminalia brownii* bark, the MIC varied as follows: Clinical *S. aureus* - 3.13 mg/ml, *V. cholerae* – 12.50 mg/ml, *Proteus mirabilis* (isolate 10) – 6.25 mg/ml) and *P. aeruginosa* (isolate 11) – 50 mg/ml.

Table 4: MIC of plant extracts against test isolates

BACTERIA	**Plant extracts (mg/ml)**				
	MVB (Acetone)	**TBB (Hexane)**	**TBB (Methanol)**	**TBB (Water)**	**ACB (Methanol)**
S. aureus ATCC 25923	3.13	6.25	6.25	6.25	-
Clinical *S. aureus*	12.50	3.13	6.25	-	50
V. Cholerae	6.25	12.50	6.25	12.50	-
Sh. Dysenteriae	3.13	-	3.13	6.25	-
Proteus spp. (isolate 7)	3.13	-	6.25	-	-
Pr. mirabilis (isolate 10)	-	6.25	-	12.50	25
P. aeruginosa (isolate 11)	25	50	-	-	-
S. aureus (isolate 6)	-	-	-	6.25	12.50
E. coli ATCC 25922	-	-	-	6.25	50
Clinical *E. coli*	-	-	-	12.50	-
Sh. flexneri (isolate 12)	12.50	6.25	6.25	25	25
E. coli (isolate 14)	-	12.50	-	-	50

Key: - Not tested; MVB: *Melia volkensii* bark; TBB: *Terminalia brownii* bark; ACB: *Albizia coriaria* bark

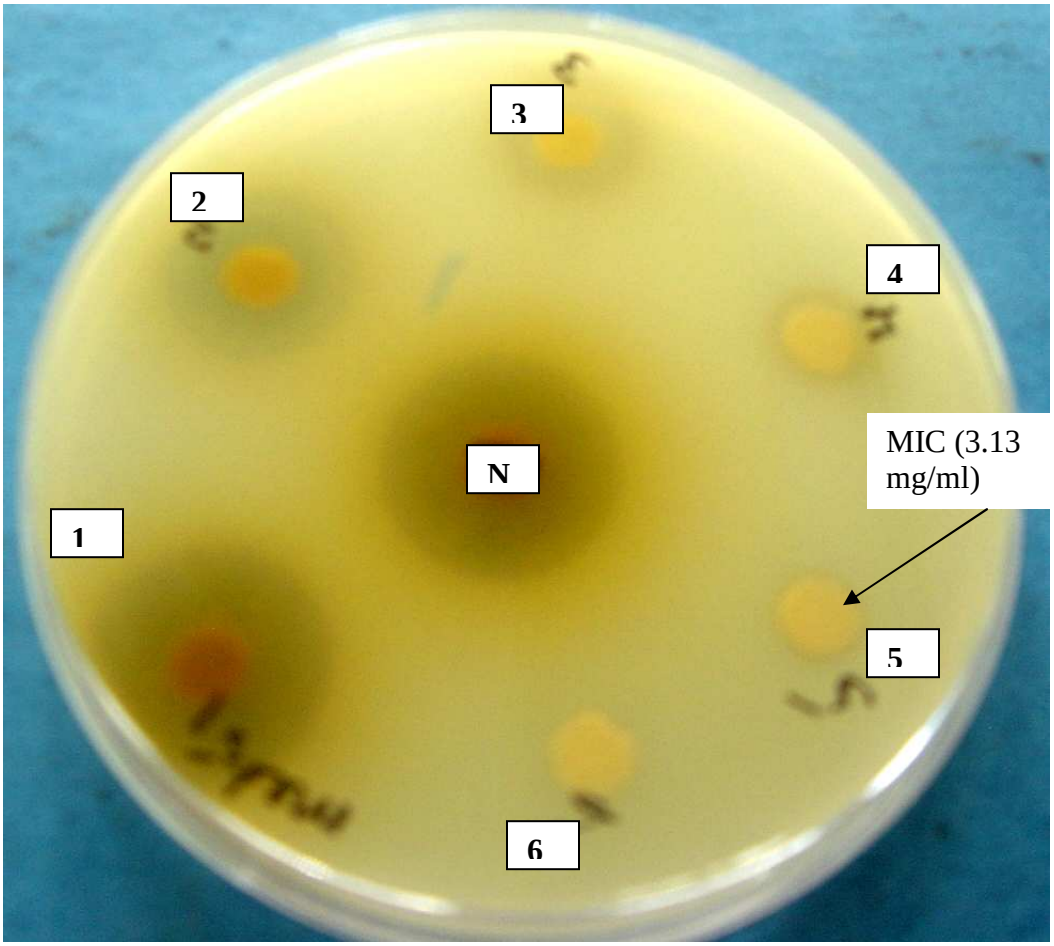

Plate 3: Determination of MIC for *Terminalia brownii* bark methanol extracts against *Shigella dysenteriae.* The different disks contain different concentrations of the extracts; (N) - 100 mg/ml; (1) - 50 mg/ml; (2) - 25 mg/ml; (3) - 12.5 mg/ml; (4) – 6.25 mg/ml; (5) - 3.13 mg/ml and (6) - 1.57 mg/ml

4.2.4 Synergistic interaction of plant extracts with antibiotics on resistant bacterial strains

The synergism tests revealed possible synergism with the antimicrobial drugs tested namely, Ampicillin, Cefuroxime and Gentamicin (Table 5). Water, hexane and methanol extracts of *Terminalia brownii* and acetone extracts of *Melia volkensii* bark demonstrated synergistic interaction with Ampicillin against *V. cholerae*. However, there was no synergism between *Terminalia brownii* (water extracts) and Ampicillin against *Shigella spp*., *Proteus mirabilis* and *Pseudomonas aeruginosa*. *P. aeruginosa* was also resistant to the combination of acetone extracts of *Melia volkensii* barks and Ampicillin. *Proteus mirabilis* was resistant to the combination of Cefuroxime and *Terminalia brownii*. Resistance was also observed on the combination of acetone extracts of *Melia volkensii* leaves and Cefuroxime by *Proteus mirabilis*, which was also resistant to the combination of hexane extracts of *Terminalia brownii* barks and Ampicillin. Synergism was however observed between hexane extracts of *Terminalia brownii* barks and Gentamicin. *Enterobacter aerogenes* was resistant to the combination of acetone extracts of *Melia volkensii* leaves (12.5 mg/ml) and Cefuroxime.

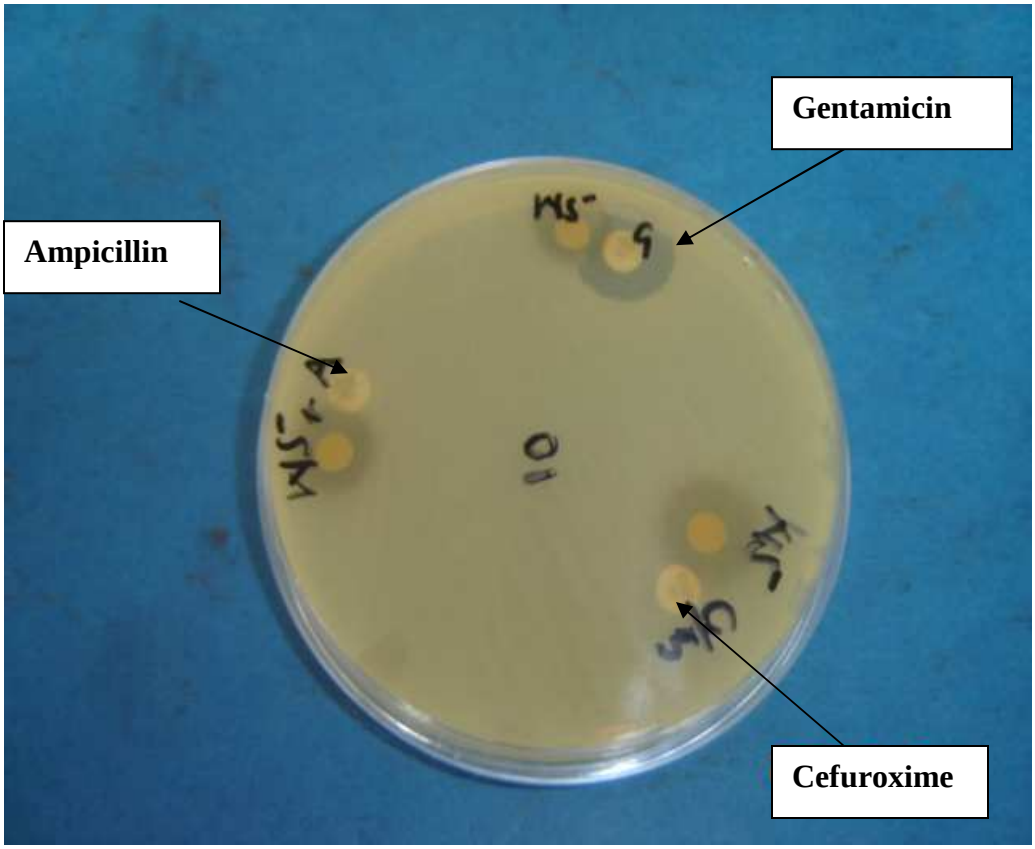

Plate 4: Synergism between *Terminalia brownii* bark water extracts with antibiotics against *Proteus mirabilis* (isolate 10)

Key: A- Ampicillin; G- Gentamicin; CXM- Cefuroxime; W5- Water extract from *Terminalia brownii* barks

Table 5: Synergism test results by disc diffusion method

ORGANISM	Plant extract (A)	rA	Drug (B)	rB	Combined rD=rA+rB	rC	Synergism
Vibrio cholerae	TBB (Water)-12.5 mg/ml	7.5	Amp	6	13.5	20	(+)
	TBB (Hexane)-12.5 mg/ml	9	Amp	6	15	20	(+)
	TBB (Methanol)-6.25 mg/ml	11.5	Amp	6	17.5	21	(+)
Shigella dysenteriae	TBB (Water)- 6.25 mg/ml	7.5	Amp	5	12.5	12.5	(-)
	MVB (Acetone)-3.125 mg/ml	7.5	Amp	5	12.5	18	(+)
	TBB (Methanol)-6.25 mg/ml	9	Amp	5	14	22	(+)
E. aerogenes (isolate 2)	TBB (Water)-50 mg/ml	4	CXM	6	10	23	(+)
	MVL (Acetone)-12.5 mg/ml	5	CXM	6	11	11	(-)
Proteus mirabilis (isolate 10)	TBB (Water)-12.5 mg/ml	5	Amp	3	8	8	(-)
	"	5	GN	3	8	20.5	(+)
	"	5	CXM	3	8	8	(-)
	TBB (Hexane)-6.25 mg/ml	7.5	Amp	3	10.5	10.5	(-)
	"	7.5	GN	3	10.5	23	(+)
	"	7.5	CXM	3	10.5	10.5	(-)
P. aeruginosa (isolate 11)	TBB (Hexane)-50 mg/ml	5	Amp	3	8	8	(-)
	"	5	CXM	3	8	25	(+)
	MVB (Acetone)-25 mg/ml	6.5	Amp	3	9.5	9.5	(-)
	"	6.5	CXM	3	9.5	26	(+)
E. coli (isolate 14)	TBB (Hexane)-25 mg/ml	4	Amp	3	7	14.5	(+)
	"	4	SXT	3	7	17.5	(+)
	"	4	CXM	5	9	16	(+)

KEY: rA/rB= Radius of zone of inhibition (mm); rC=Distance between centers (enlargement of zone size); rC>rD=Synergism (+); rC=rD=Neutralism (-); Amp – Ampicillin; CXM – Cefuroxime; GN – Gentamicin; SXT - Cotrimoxazole

4.2.5 Kill kinetics of plant extracts

The kill kinetics of the different plant extracts against *S. aureus* and *E. coli* were evaluated under minimum bactericidal concentrations (Table 6). Different solvent extracts show different levels of activity. Hence, the plant extracts that were highly active were used in this assay to show different rates of bactericidal effect. The rates of killing of the extracts are shown in figures 4 and 5.

Table 6: Concentrations used for kill kinetics bioassay

Extract	**Concentration (MBC)**	**Organism**
Terminalia brownii-bark (Hexane)	100 mg/ml	*S. aureus*
Terminalia brownii-bark (Methanol)	25 mg/ml	*S. aureus*
Melia volkensii-bark (Acetone)	100 mg/ml	*S. aureus*
Albizia coriaria-bark (methanol)	25 mg/ml	*E. coli*
Melia volkensii-leaves (Acetone)	100 mg/ml	*E. coli*

The growth curve of *Staphylococcus aureus* with *Terminalia brownii* (hexane extract) showed more than 50 % decrease in the number of viable cell count (colony forming units) in the first 4 h, then a slight increase in the number colony forming units after 8 h, after which there was a sudden decrease in the number

colony forming units. After 24 h, there was a complete reduction in the number of viable cells. Methanol extracts of *Terminalia brownii* also showed more than 50% reduction of the number of colony forming units after 24 h. *Melia volkensii* bark (acetone) extracts were inhibitory for the first 8 h after which the number of colony forming units increased. However, the population of the control culture of *S. aureus* constantly increased during the entire test period (Figure 4).

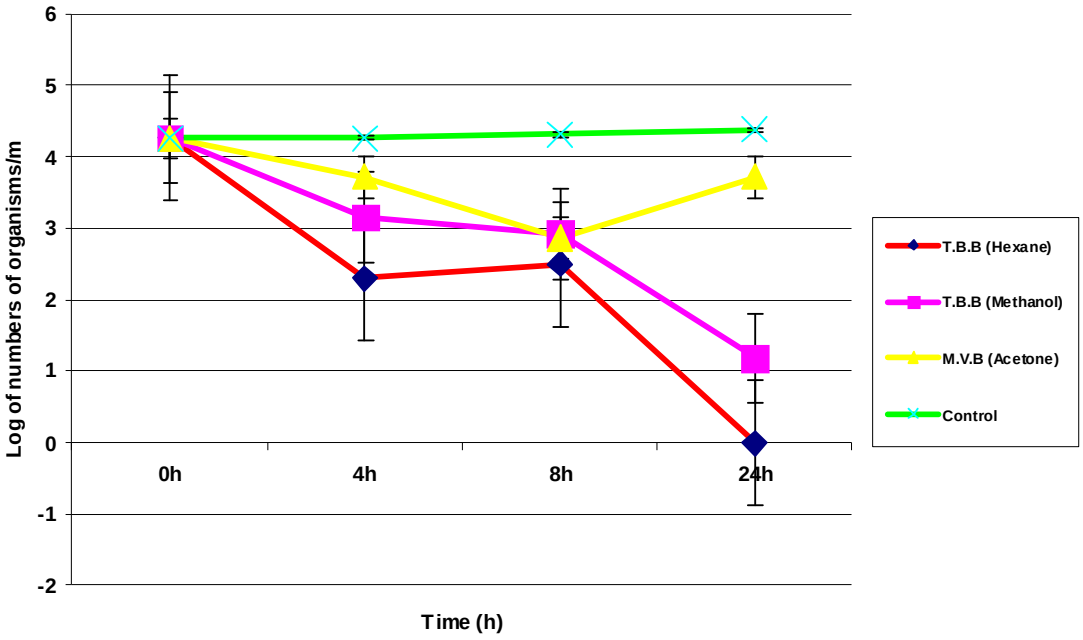

Figure 4: Kill kinetics of *Terminalia brownii* bark and *Melia volkensii* bark extracts against reference strain of *S. aureus*

The growth curve of *Albizia coriaria* (methanol extracts) indicated a constant decrease in the number of colony forming units for the entire test period. However, there was no complete reduction in the number of colony forming units after 24 hours, since the number of colony forming units was reduced to or by at least 50 %. A similar pattern was observed with acetone extracts of *Melia volkensii* leaves. The population of the control cultures of *E. coli* constantly increased during the entire test period (Figure 5).

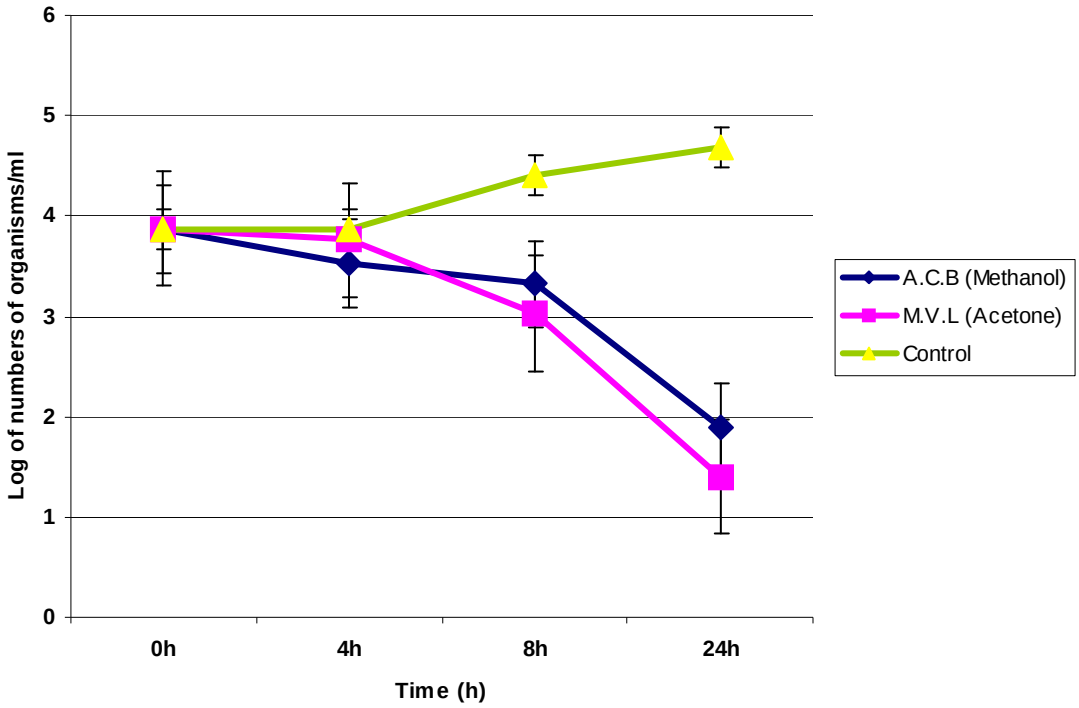

Figure 5: Kill kinetics of *Albizia coriaria* bark and *Melia volkensii* leaf extracts against reference strain of *E. coli*

4.3 Phytochemical analysis of plants

Preliminary phytochemical analysis of the plant extracts indicated presence of different phytochemicals such as tannins (plate 4), which was indicated by the formation of blackish-blue colour after mixing the crude extract in 1 ml of water and gelatin salt reagent. The results obtained from the TLC tests corresponded with the preliminary phytochemical test results (Table 7). *Terminalia brownii* contained high amounts of phenolics, steroids, anthraquinones, tannins and saponins (Table 7). *Melia volkensii* extracts contained glycosides, phenols, steroids, flavonoids, triterpenoids, tannins and saponins. *Albizia coriaria* contained high amounts of alkaloids and saponins. All the plants in this study contained tannins which are effective in controlling diarrhoea, except for *Aloe secundiflora* which had the least phytochemicals, namely, sterols and saponins, in very small amounts.

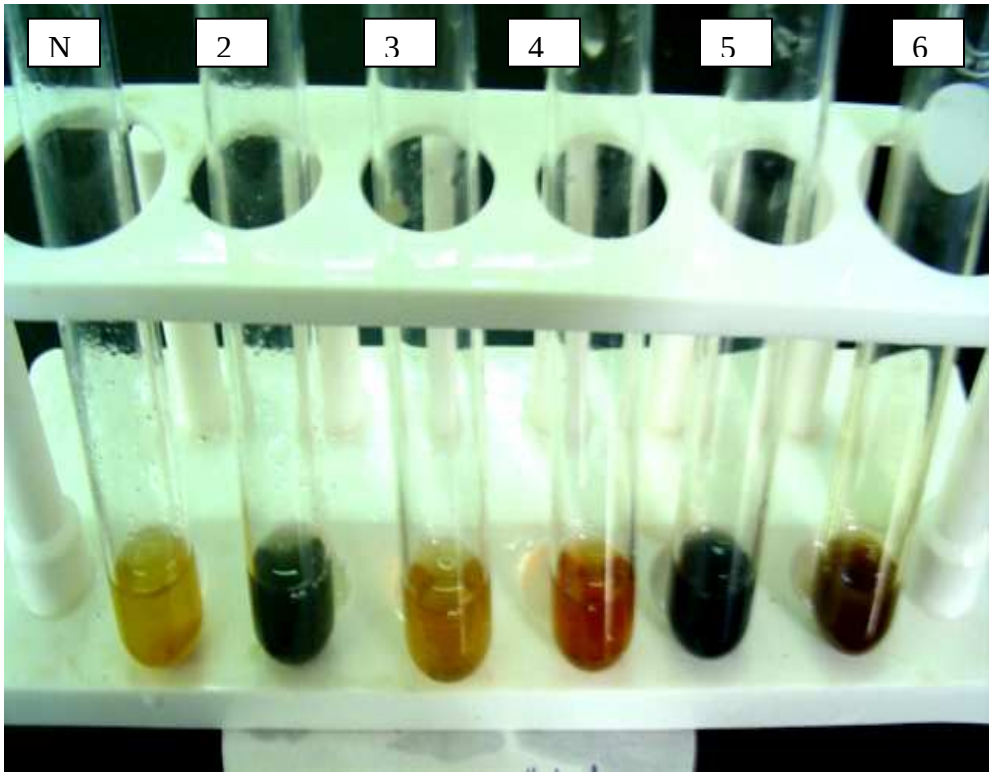

Plate 5: Preliminary phytochemical tests of tannins for (2) – *Melia volkensii* leaves; (3) – *Aloe secundiflora* leaves; (4) – *Melia volkensii* bark; (5) – *Albizia coriaria* bark; (6) – *Terminalia brownii* bark; N – Negative control

Table 7: Profile of phytochemicals present in plant extracts

Chemical group	***Terminalia brownii* (stem bark)**	***Melia volkensii* (stem bark)**	***Melia volkensii* (leaves)**	**Aloe secundiflora**	***Albizia coriaria* (stem bark)**
Cardiac glycosides	-	+	-	-	-
Phenolics	+++	-	+	-	-
Steroids	+++	+	++	-	+
Sterols	+	+	-	+	-
Anthraquinones	++	-	-	-	-
Flavanoids	+	+	+	-	+
Triterpenoids	-	+	-	-	+
Galloyl tannins	+++	-	-	-	-
Catechol tannins	+++	-	+	-	+
Alkaloids	+	-	-	-	++
Saponins	++	+	+	++	+++

Key: - Absent; + Trace; ++ Present in appreciable quantity; +++ Present in large amounts

4.4 Toxicity screening

Water extracts were used for toxicity screening because the local people mostly use water extraction to obtain their medicine. The LD_{50} values that were found to be less than 30 µg/ml were considered toxic while those above 100 µg/ml were considered non-toxic. Hence values between 30 µg/ml and 100 µg/ml were considered a safe dose regime (Saupe, 2005). The results showed that all the extracts were non-toxic to the shrimps. Their LD_{50} values were greater than 100 µg/ml and hence they had very low toxicity. The LD_{50} values for the plant extracts ranged between 39.42 µg/ml-1930.70 µg/ml. *Terminalia brownii* exhibited the least toxicity (1930.70 µg/ml) while *Melia volkensii* leaves exhibited the highest toxicity (39.42 µg/ml). However, the LD_{50} value for *Melia volkensii* leaf extract was between 30 µg/ml and 100 µg/ml (Table 8), which are considered a safe dose regime.

Table 8: LD_{50} values for the plant extracts`

PLANT	**LD50 VALUES (µg/ml)**
Terminalia brownii	1930.70
Melia volkensii (stem bark)	187.38
Melia volkensii (leaves)	39.42
Aloe secundiflora	416
Albizia coriaria	533.67

CHAPTER 5

5.0 DISCUSSION

The results from the antibiotic sensitivity patterns revealed that the environmental isolates showed greater drug resistance as compared to the clinical isolates (Figure 1). In his study, Faruque *et al*., (2002) showed that environmental strains of *Shigella dysenteriae* Type 1 isolated from surface waters in Bangladesh were resistant to one or more antibiotics. In the present study, none of the clinical isolates were multi-drug resistant and the only resistance shown was to Ampicillin alone by *Vibrio spp* and *Shigella dysenteriae*. Multi-drug resistance was shown by *Proteus mirabilis* (Isolate 10) to Ampicillin, Gentamicin and Cefuroxime and *Escherichia coli* (Isolate 14) to Ampicillin, Cotrimoxazole and Cefuroxime. *E. coli*, is already known to be multi-drug resistant (Nascimento *et al*., 2000; Kariuki and Hart, 2001; Ndun'gu *et al*., 2004). High rate of resistance to Ampicillin was observed among the test isolates. These antibiotic resistance results indicate that there was resistance to first-line, broad-spectrum antibiotics. This resistance may be because Ampicillin is a first-line antibiotic for treatment of infections due to enteric pathogens and is also generally inexpensive and hence accessible to more people (Okeke *et al*., 1999). The presence of multi-drug resistant strains in environmental waters is a public health problem since these strains may serve as reservoirs of drug-resistant genes (Faruque *et al*., 2002). About half of the tested environmental isolates were resistant to the tested antibiotics and these results indicate that people in the Lake Victoria region are at risk of being infected by

drug-resistant pathogens from the environment. The higher resistance among environmental isolates may have been due to indiscriminate sewage ad hospital waste disposal into riverine areas. This exposes the environmental bacteria to antibiotic. With time, the envronmental pathogens may acquire resistance to the antibiotics. Sewage waste may also contain bacteria that are already drug-resistant and when these bacteria interact with the environmental pathogens, there maybe transference of drug-resistant genes that will confer resistance to the previously susceptible environmental pathogens. These resistance problems demand that a renewed effort be made to seek antibiotic agents effective against pathogenic bacteria that are resistant to current antibiotics.

Many of the traditionally used medicinal plants have been investigated scientifically for antimicrobial activity and a large number of plant products have been shown to inhibit growth of pathogenic bacteria (Devi *et al*., 2002; Agbor *et al*., 2004; Ahmad and Aqil, 2006; Oben *et al*., 2006; Palombo *et al*., 2006; Venkat *et al*., 2006).. The results in the present study show that most of the test isolates are susceptible to plant extracts. However, some plant extracts (*Aloe secundiflora*) were inactive against some bacteria as indicated by the results of this study. The results of *Pseudomonas aeruginosa* (isolate 13) are consistent with the results of a study by Parekh and Chanda, (2006) that showed that the organism was resistant to plant extracts. This bacterium is known to be drug-resistant. It has been suggested that this resistance to plant extracts may be due to the presence of the

outer membrane of the bacterial cell wall, which acts as a barrier to various environmental factors such as antibiotics or due to the differences in the cell wall composition of various bacteria (Tiwari *et al*., 2005). Some plants only act on gram-positive cell wall while others act on gram-negatie cell walls. These differences in cell wall composition protect the pathogens from the plant extracts, depending on the mode of action of the plant. The resistance to *Aloe secundiflora* indicates that although some plants are used in the management of diarrhoea, not all prescribed antidiarrhoeal medicinal plants may be effective against enteric pathogens. However, this resistance does not rule out the potential of the plant as antibacterial agents for treatment of enterobacterial infections. The plant may be effective while in combination with other medicinal plants.

There was variation in the MICs of each plant extract within each particular microorganism (Table 4). The variation was also dependent on a particular microorganism. This may be because the test isolates have different levels of intrinsic tolerance to antimicrobials (Ahmad and Aqil, 2006). These different levels of variation show that variation of MIC is based on particular bacteria and on a particular medicinal plant. The variation within a particular medicinal plant may have been due to the differences in the amount of phytochemicals in each particular plant.

The antidiarrhoeal medicinal plants had varying levels of activity against the enteric bacteria in all the solvents used for extraction. The potency of the fractions was in order of acetone> methanol>hexane>water. For instance, the solvent extracts of *Terminalia brownii* bark showed varied levels of potency against *Shigella flexneri* (isolate 12) in the disc diffusion assay. The zones of inhibition of the *Terminalia brownii* bark extracts ranged as follows: acetone (23 mm), methanol (19 mm), hexane (15 mm) and water (10 mm) (Appendix 8). The differences in the potency may be due to polarity of the solvents (Eloff *et al*., 2005). It is known that solvents with high polarity have affinity to solvents of high polarity compounds and hence, they are able to extract high polar phytochemicals which. The results show that the solvents with high polarity are the ones that extracted more bioactive compounds. Previous studies have shown that plant extracts in organic solvents provided consistent antimicrobial activity as compared to those extracted in water (Devi *et al*., 2002; Fyhrquist *et al*., 2002; Pessini *et al*., 2003; Durmaz *et al*., 2006; Parekh and Chanda, 2006). In the present study, aqueous extracts did not provide much activity as compared to the extracts from organic solvents. However, the antimicrobial activities by water extracts authenticate the traditional use of the tested medicinal plants for their therapeutic values. It was also evident that different solvents produce different levels of activity from medicinal plants.

Varying levels of potency were also observed in all the plants used. The variation was in the order of *Terminalia brownii* barks>*Melia volkensii* barks>*Albizia coriaria* barks>*Melia volkensii* leaves>*Aloe secundiflora*. This may be due to the presence of different types of phytochemicals in varying concentrations. Tannins, flavonoids, alkaloids, saponins, reducing sugars, sterols and triterpenes have been reported to have antidiarrhoeal as well as antibacterial activity (Lewis, 2003; Palombo, 2006). For instance, flavonoids have an ability to inhibit intestinal motility and hydro-electrolytic secretion, inhibit the intestinal secretory response induced by prostaglandin E_2 and have antioxidant properties responsible for the inhibitory effects exerted upon several enzymes, including those involved in the arachidonic acid metabolism (Venkat *et al*., 2006). The antidiarrhoeal activity of the phytochemical compounds has been attributed to their antimicrobial activity (Mamtha *et al*., 2004; Brijesh *et al*., 2006).

Terminalia brownii barks had the highest amount of tannins (Table 7). Tannins have been known to be effective in the prevention of colonization of enteric pathogens and consequently control diarrhoea (Devi *et al*., 2002; Mamtha, 2005; Oben *et al*., 2006; Palombo, 2006). These substances also precipitate proteins of the erythrocytes, reduce peristaltic movement and intestinal secretion (Venkat *et al*., 2006). The plant also contained flavonoids, steroids, alkaloids, and saponins, all of which have been known to have antidiarrhoeal activity (Galvez *et al*., 1993; Agbor *et al*., 2004; Brijesh *et al*., 2006; Palombo, 2006) through inhibiting the

growth of the enteric bacteria that cause diarrhoea. The antibacterial activity of the phytochemicals could be the reason why *Terminalia brownii* bark extracts had the highest activity against the enteric pathogens. Previous studies have shown presence of antibacterial phytochemicals in *Terminalia brownii* (Mbwambo *et al*., 2007). Likewise, previous studies have shown that antidiarrhoeal medicinal plant extracts can be used to treat diarrhoea due to enteric pathogens (Kokwaro, 1976; Mbwambo *et al*., 2007). *Melia volkensii* and *Albizia coriaria* extracts also contained phytochemicals, which have antibacterial activity (Nascimento *et al*., 2000; Mamtha *et al*., 2004; Ahmad and Aqil, 2006; Brijesh et al., 2006). *Aloe secundiflora* had low activity against the isolates, since it only contained saponins and sterols, in very small amounts. This may be the reason for its low activity. The causes of the variation of potency in the plants may be due to the variation of phytochemical compounds present in the plants.

Of concern is the number of bacteria that have become resistant to ampicillin necessitating combination therapy or use of other antibiotics. Previous studies have indicated that antibiotic-resistant bacteria are becoming a threat to human health (Kariuki *et al*., 2000; Faruque *et al*., 2002; Davis *et al*., 2005 and Lin and Biyela, 2005). The present study revealed that the tested plants have great therapeutic potential. It also revealed the importance of combination of plant extracts with antibiotics to which the bacteria were resistant. For instance, *E. coli* (isolate 14) was resistant to Ampicillin, Cotrimoxazole and Cefuroxime, but there

was increased susceptibility to the drugs when they were combined with hexane extracts of *Terminalia brownii* stem barks. The interaction of plant extracts with antibiotics may be due to novel mechanisms other than those already known (Nascimento *et al*., 2000; Ahmad and Aqil, 2006). Other studies have reported synergistic interaction between plant extracts and antibiotics to which a particular microorganism is resistant (Nascimento *et al*., 2000; Aqil *et al*., 2005; Ahmad and Aqil, 2006; Betoni *et al*., 2006). Therefore, synergistic effect of plant extracts and antibiotics is important in the control of antibiotic-resistant bacteria. These results may be useful in providing data necessary in the process of incorporating herbal medicine in conventional health care services.

The kill kinetics results indicated that hexane extracts of *Terminalia brownii* were bactericidal against *S. aureus* since there was a three-fold reduction of bacterial concentration in the treated cultures after the 24-h test period. However acetone extracts of *Melia volkensii* bark were bacteriostatic against *S. aureus* since there was no complete reduction of viable colony forming units after the 24-h test period. Other studies have shown similar trends (Woolfrey and Enright, 1990; Okemo *et al*., 2004). This may be because the *E. coli* strains may have reverted to "L" forms of the organisms or due to the mode of action of the extracts, cell membrane permeability or genetic factors (Woolfrey and Enright, 1990). *Melia volkensii* leaves (acetone extracts) and *Albizia coriaria* barks (methanol extracts) were bactericidal against *E. coli* since there was a three-fold reduction of bacterial

concentration in the treated cultures after the 24 h test period. From the study, it was also evident that gram-positive organisms were completely killed after 24 h unlike the gram-negatives and this gives an indication of the mode of action of the medicinal plants that were tested. The bactericidal effect of *Terminalia brownii* against *S. aureus* is important since this bacterium has been ranked as the 2nd most common cause of nosocomial infections in immunocompromised individuals (Wertheim *et al*., 2005). This inhibition by *Terminalia brownii* may be an indication for successful treatment of nosocomial infections in high-risk groups of people.

The cytotoxicity of a compound is important in the development of therapeutic agents, since the antibacterial agents should not be toxic to the host. A plant may have a high antibacterial activity, but if it were toxic, it would be detrimental to the host. From this study, it is evident that *Terminalia brownii* stem bark extracts, which showed the highest activity against the test isolates, also exhibited a relatively low toxicity on brine shrimps while *Melia volkensii* leaf extracts had the highest toxicity and yet it had showed the least antimicrobial activity against the enteric pathogens. The cytotoxicity results of *Terminalia brownii* stem bark are consistent with a study by Mbwambo *et al*., (2007), which showed that the roots and stem bark of *Terminalia brownii* exhibited relatively mild cytotoxicity activity against brine shrimp larvae with LD_{50} values ranging from 113.75–4356.76 and 36.12–1458.81 µg/ml, respectively. Hence, the plant extracts tested

in this study are generally non-toxic and this shows that they may be suitable for the treatment of human diseases and be able to avoid possible detrimental health risks. *Melia volkensii* leaf extracts had a higher toxicity (39.42 µg/ml) than its bark extracts (187.38 µg/ml) and this indicates that the bark extracts are preferable to the leaf extracts in the management of diarrhoea. However, leaves can be used as a substitute to the bark extracts for conservation purposes.

Few ethnobotanical studies have been conducted in Kenya (Njoroge, 1992; Kokwaro, 1993; Kisangau, 1999; van Wyk, 2002). None of them provided ethnobotanical data from Rachuonyo district in Kenya, thus providing a basis for more ethnobotanical studies in Kenya. It has been shown that ethnobotanically derived phytochemicals have greater activity than compounds derived from random screening and therefore a greater potential for products developed (Balick and Cox, 1996; Flaster, 1996). This claim has been supported by this study, which showed that three of the plants, which were ethnobotanically derived, had antimicrobial activity and hence the knowledge of plants frequently used in the treatment of diarrhoea in Rachuonyo district has scientific proof. However, one plant, *Aloe secundiflora* showed low activity despite being ethnobotanically derived. During the ethnobotanical survey, most people frequently mentioned that the plant was used in the treatment of diarrhoea. The plant may be effective in treating only stomachache or treating diarrhoea when it is used in combination with other medicinal plants. Hence measures are necessary to inform the local

people of Rachuonyo district of this theory. There was correlation between the FL values of the selected medicinal plants and their antimicrobial activities. The plants had high FL values and exhibited antimicrobial activity, which shows that plants with a higher FL value, has a higher chance of having antimicrobial activity and higher chance of being effective in the treatment of diarrhoea.

5.1 Conclusions

Data obtained from the ethnobotanical survey has indicated that knowledge obtained from traditional communities is an indication of the antimicrobial activity of medicinal plants.

This study has shown that both the organic and aqueous extracts obtained from *Terminalia brownii, Melia volkensii* and *Albizia coriaria* have demonstrated antimicrobial activity against enteric microorganisms and therefore, they can be used in the treatment of diarrhoea diseases, especially those caused by antibiotic-resistant enteric microorganisms. Varying levels of activity were observed within the medicinal plants under study.

It has also been demonstrated through the synergism tests that extracts from *Terminalia brownii* and *Melia volkensii* can increase the potency of antibiotics to which the microorganisms are resistant. This synergism provides new alternative treatment for infectious diseases, enabling the use of the particular antibiotic when

it is no longer an effective therapeutic treatment on their own during antibiotic therapy. The results in this study may provide data necessary in the process of incorporating herbal medicine in conventional health care services.

The phytochemical tests done on the medicinal plants in this study have indicated that phytochemical extraction is best done using organic solvents rather than water to obtain most of the phytochemicals (active compounds). The tests also revealed that the medicinal plants contained phytochemicals with antibacterial activity.

The extracts from *Terminalia brownii*, *Albizia coriaria* and *Melia volkensii* were found to be bactericidal to *S. aureus* and *E. coli*. They were also generally non-toxic to brine shrimps and therefore they might be non-toxic to human beings. Thus they can be used in the treatment of diarrhoea due to enteric pathogens and avoid the possible detrimental health risks.

The antibiotic susceptibility tests revealed that there is an increasing rate of multi-drug resistance among environmental enteric pathogens. This poses a great health risk for people living in the Lake Victoria region since they are at risk from being infected with multi-drug resistant pathogens. In turn, this may create a public health situation.

Lastly, but not least, the laboratory evaluation of the antidiarrhoeal medicinal plants used in this study has provided scientific support for their wide application in traditional medical practices, except for the use of *Aloe secundiflora* in the treatment of diarrhoea. This shows that not all prescribed medicinal plnats are effective against enteric pathogens. However, this plant may be effective only as a pain reliever for stomachache in human beings or it may be an effective antidiarrhoeal agent in combination with other medicinal antidiarrhoeal medicinal plants.

5.2 Scope for further studies

Although this study has provided useful data concerning the antimicrobial activities of *Terminalia brownii* barks, *Melia volkensii* barks, *Albizia coriaria* barks, *Melia volkensii* leaves and *Aloe secundiflora*, further work is necessary to provide more data. For instance;

- Further studies on the mode of action of extracts from *Terminalia brownii* barks, which had the highest activity against the enteric bacteria, and the other active extracts should be done.
- Those plants showing very low MIC values (6.25 mg/ml or less) should be further evaluated for their effect against known drug-resistant pathogens.
- The plant extracts' toxicity should be further evaluated *in vivo*, to be able to determine the safest dosage for the treatment of diarrhoea in humans.

- Further phytochemical analysis of the plants should be done to isolate and characterize the specific compounds responsible for the antidiarrhoeal/antibacterial activity as well as for the synergistic effect with antibiotics and to evaluate the molecular basis of the synergistic interactions of the plants
- Further *in vivo* studies are needed to confirm the antibacterial activity results obtained in this study.
- Since only a few isolates were tested, further studies involving a large number of resistant pathogens are necessary to draw meaningful conclusions.

5.3 Recommendations

- The use of herbal medications together with antibiotics should be encouraged in order to avoid the problem of drug resistance
- Measures should be made to domesticate and conserve the antidiarrhoeal medicinal plants to increase their availability to herbalists and other users.
- The government should renew its efforts in combating the problem of indiscriminate industrial and domestic sewage discharge into the environment. This will reduce the problem of contamination and hence improve the health of the people while at the same time, avoiding infections due to drug-resistant pathogens.

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APPENDICES

Appendix 1: Questionnaire

Name:.....Gender (M/F):.....Age:.....

Education level…… Occupation…….

Origin: District..... Division..... Village..... Date…….

1. Which plants do you consider as important sources of medicines for the treatment of diarrhoea among the people of this region?
2. Starting with the most important, please rank these in order of utilization (Highly utilized ones first)
3. What other diseases do you treat using the same plants?
4. Please state how they are utilized:

Plant name	Part used	Method of preparation	Dosage	Other mixtures to it

5. Do you use these plants for: self-medication, treating other or selling?

6. Have you planted any of them?

7 Are the plants used for treating diarrhoea still easily available here? (Yes/No)

Appendix 2: Number of respondents in each division of Rachuonyo District

Division	**No. of Male**	**No. of Female**	**Total**
West Karachuonyo	9	24	33
East Karachuonyo	12	43	55
Kasipul	17	57	74
Kabondo	6	23	29
			Grand Total=191

Appendix 3: Biochemical tests for identification of enteropathogens

Carbohydrate fermentation with production of acid and gas:

TSI medium is used for this test. It is a non-selective medium that indicates some combination of fermentation reactions by means of a phenol red indicator dye. It also reveals gas and hydrogen sulphide production. Bacteria that produce hydrogen sulfide reduce thiosulfate in the media. Hydrogen sulphide is indicated by a reaction of ferric chloride to form a black precipitate of ferrous sulfide.

Indole production:

It is tested using Sulphur Indole Motility (SIM) media. The indole test indicates the capacity of an isolate to cleave a compound called indole from amino acid tryptophan. To test if cleaving occurs, a few drops of Kovac's reagent are added

and a bright red ring will be formed at the surface of the tube. If it is negative, the Kovac's remains yellow. The media also tests for hydrogen sulphide production and motility.

Citrate utilization:

This is tested using Simmon's citrate medium. It contains citrate as the only usable carbon source and a pH indicator, bromothymol blue. If an isolate can utilize citrate, it grows and produces alkaline by-products, resulting in a conversion of the medium's colour from green (neutral) to blue (alkaline).

Urease test:

Urea medium contains 2% urea and phenol red pH indicator. Some bacteria produce the enzyme urease, which hydrolyses urea into 2 molecules of ammonium and carbon dioxide. Ammonium raises the pH of the medium, and the indicator turns from yellow to bright pink/red in colour.

Methyl-Red (MR) test:

This test indicates a form of glucose fermentation in which large amounts of mixed acids accumulate in the medium. The pH is lowered to around 4.2, so that methyl-red dye remains red when added to the tube. Methyl-red negative bacteria do not lower the pH to this degree and the tube is yellow to orange in the presence of the dye.

Voges-Proskauer (VP) test:

It determines whether the product of glucose fermentation is a neutral metabolite called acetyl-methylcarbinol (acetoin). This substance is detected by adding Barrit's reagent, which forms a pink complex in 30-60 minutes. With negative results the tube remains brown-yellow in colour.

N/B: Enteric pathogens that are MR-positive are usually VP-negative.

Motility test:

This is done using the hanging drop slide technique by observing the microscopic behaviour of the cell. In the true motility, the cell swims and progresses from one point to another. Non-motile cells oscillate in the same relative space (Brownian movement), but they do not swim from one point to another.

Oxidase test:

This laboratory test is based on detecting the production of the enzyme cytochrome oxidase by Gram-negative bacteria. It is used to discriminate between aerobic Gram-negative organisms like *Pseudomonas aeruginosa* and other *Enterobacteriaciae*. A colony is picked for testing using a sterile wooden toothpick. It is transferred to the surface of a slide. The test slide surface is impregnated with the reagent tetramethyl-*p*-phenylenediamine dihydrochloride. This reagent causes a dark purple color to appear in the presence of cytochrome oxidase. The reaction color will change from pink to maroon to dark purple. The

test results should be read within 10 seconds. Some organisms may show slight positive reactions after this period and such results are NOT considered definitive.

Appendix 4: Reaction of Enteropathogens in tube media

	TSI			LIM							
Bacteria	**Butt**	**Slant**	**H_2S**	**Lys**	**Ind**	**Mot**	**SC**	**Urea**	**MR**	**VP**	**OX**
Escherichia	+	+	-	+	+	+	-	-	+	-	-
Klebsiella	+	+	-	+	V	-	+	+	-	+	-
Enterobacter	+	+	-	V	-	+	+	-	+	+	-
Proteus	+	-	-	-	V	+	V	+	+	-	-
Citrobacter_	+	-	V	-	V	+	+	V	+	-	-
Providencia	+	-	-	-	+	+	+	+	+	-	-
Morganella	+	-	-	V	+	+	-	+	+	-	-
Salmonella	+	-	+	+	-	+	-	-	+	-	+
Shigella	+	-	-	-	+	-	-	-	+	-	-

TSI- Triple Sugar Iron

LIM- Lysine Indole Motility

H_2S- Hydrogen sulfide

Lys- Lysine

Ind- Indole

Mot- Motility

Sc- Simmon's citrate

MR- Methyl Red

VP- Voges proskauer

OX-Oxidase

+- positive

-_Negative

v- Variable among strains

Appendix 5: Phytochemical tests

Alkaloids:

The extract is boiled for 15 minutes in 25ml of 1% HCL. Equal volumes of the resulting suspension are filtered in two test tubes (A and B). To A, 5 drops of freshly prepared Dragendorf's reagent is added. Formation of a precipitate indicates the presence of alkaloids. To confirm the results, B is treated with saturated sodium carbonate solution until a drop of the solution turns the Universal indictor paper, blue (pH 8-9). The resulting solution is dissolved in 4ml chloroform and allowed to stand. The aqueous layer is collected and acetic acid is added to it dropwise, until the solution turns Universal indicator paper yellow-brown (pH 5).

Saponins:

The extract is added to 15ml water and warmed on a water bath for 15 minutes. The resulting solution is filtered and left to cool to room temperature and then 10ml is transferred in a test tube. This is shaken thoroughly for 10 seconds and the height of the persistent (5-10 minutes) honeycomb froth is measured. Honeycomb froth higher than 1 cm is a confirmation for the presence of saponins. To confirm the results, 1ml of the extract is dissolved in carbon tetrachloride. 4 drops of concentrated sulphuric acid and a drop of acetic anhydride are added to 1 ml of

the filtrate. Appearance of blue-green or red-brown colour often accompanied by formation of a pink ring is a confirmation for the presence of saponins.

Flavonoids:

Each extract is added to 10ml of water and 5 ml methanol. A few magnesium turnings are added to 3 ml of the mixture and followed by dropwise addition of concentrated HCL (cyaniding). Development of, orange, red or pink colours indicate presence of flavonoids.

Polyphenols:

10 ml ethanol is added to each extract and 3 ml of the resulting solution is transferred in test tubes and warmed in a water bath for 15 minutes. 3 drop of freshly prepared ferric cyanide solution is added to the extract solution. Formation of a blue-green colour indicates presence of polyphenols.

Tannins:

1ml of the crude extract is dissolved in water and then tested with gelatin salt reagent containing (1% gelatin and 10% sodium chloride). Allow tannins are indicated by the presence of a blackish-blue colour. Catechol tannins are indicated by a greenish-black coloration.

Steroids and sterols:

1ml of the crude extract is dissolved in 0.5ml of concentrated sulphuric acid, 0.5ml acetic anhydride and 0.5ml chloroform. A change of colour from violet to blue or green indicates presence of steroids. A red coloration indicates presence of sterols.

Triterpenes (Salkowski test):

300 mg of extract mixed with 5 ml chloroform and warmed for 30 minutes. The chloroform solution is then treated with a small volume of concentrated sulphuric acid and mixed properly. The appearance of red color indicates the presence of triterpenes.

Glycosides (Keller-Killani test):

5 ml of each extract is treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This is underlayed with 1 ml of concentrated sulphuric acid. A brown ring of the interface indicates a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout this layer.

Appendix 6: Thin Layer Chromatography

Organic Compounds - 50%Vanillin Sulphuric Acid

5ml of vanillin was added to 5ml of Sulphuric acid then sprayed onto the developed plate before heating in the oven for 10 min at 105^0C and in day light for yellow, brown, or purple spots as an indication of organic compounds.

Phenolics – 5% Ethanolic Potassium Hydroxide

Accurately 0.5g of potassium hydroxide was weighed out and dissolved into 10ml of ethanol; this was then sprayed onto the developed TLC plate and observed in day light for Phenolics indicated by red or pink spots.

Terpenoids – 0.5% Anisaldehyde

The reagent was prepared by making up 0.5ml of Anisaldehyde in a mixture of Sulphuric acid: glacial acetic acid: methanol in the ratio 5: 10: 85: respectively. This was sprayed then the plate heated in the oven at 105 ^{0}C for 10min before observing for purple / blue / red spots to indicate the presence of terpenoids.

Anthraquinones – 10% Methanolic Potassium Hydroxide (Kedde Reagent)

1g of potassium hydroxide pellets accurately weighed was dissolved into 10 ml of methanol then sprayed on the developed plate and observed for yellow or yellow brown spots as an indication of anthraquinones.

Flavonoids – Ammonia Fumes

The silica gel at the base of the developed TLC plate was scrapped off before immersing it in little ammonia in a developing tank in the fume hood. This was then left for 10 min for the ammonia fumes to diffuse to the spots. The plate was then removed and observed for presence of flavonoids indicated by yellow / blue / dark brown / red / orange or green spots.

Steroids – Liebermann-Burchard Reagent

1ml of concentrated Sulphuric acid was added to 20 ml of acetic anhydride then mixed with 50ml of chloroform. This was sprayed onto the developed TLC plate and heated in the oven at 90^0C for 15 min before observing for grey / yellow / pink spots as an indication of steroids.

Cardiac Glycosides – 20% Antimony Chloride in Chloroform (Carr-Price Reagent)

2g of antimony chloride was accurately weighed and then dissolved into 10ml of chloroform. This was then sprayed onto the developed TLC plate and then heated

in the oven at 100^0C for 10min before observing for brown spots as an indicator of presence of cardiac glycosides.

Amines – 0.5% Ninhydrin in Acetone

0.5 g of Ninhydrin was added to 100ml of acetone then sprayed onto the developed plate before heating in the oven for 105^0C for 10min. after which the plate was observed for yellow spots as an indication of the presence of secondary amines.

Alkaloids – Dragendoff Reagent

A stock solution was prepared by mixing bismuth subnitrite (1.7g) with 80ml of distilled water and glacial acetic acid (20ml). To this potassium iodide (50% w/v) was added and shaken to dissolve. A working solution was then made by mixing the stock solution (100ml) with 20ml of glacial acetic acid, in 1000ml volumetric flask, before adding distilled water up to the mark. (The solution was kept in dark bottle). The reagent was then sprayed onto the developed TLC plate before observing for orange zones on a yellow background as an indication of alkaloids.

Appendix 7: Antibiotics used for susceptibility tests

Antibiotics for Gram negative organisms (µg/ml)	**Antibiotics for Gram positive organisms (µg/ml)**
Chloramphenicol (30) - Chl	Oxacillin (1) – Ox
Cotrimoxazole (23.75/1.25) -SXT	Vancomycin (30) – VAN
Gentamicin (10) - GM	Clindamycin (30) – C
Ampicillin (10) - AM	Cotrimoxazole (23.75/1.25) – SXT
Cefuroxime (30) - CXM	Chloramphenicol (30) – Chl
Amikacin (30) -AN	Cefotaxime (30) – CTX
	Ceftazidime (30) – CAZ

Appendix 8: Antimicrobial assay results

1. Antimicrobial assay for *Terminalia brownii* barks

***Terminalia brownii* Barks**

Microorganisms	**Hexane extract**	**Methanol extract**	**Acetone extract**	**Water extract**
QC *S. aureus*	12	17	6	10
CLIN *S. aureus*	15	15	6	7
QC *E. coli*	6	6	6	14
CLIN *E. coli*	6	6	6	12
Vibrio cholerae	18	24	6	15
Salmonella typhi	6	6	6	6
Shigella dysenteriae	6	6	6	15
Enterobacter aerogenes (isolate 2)	6	18	6	8
E. coli (isolate 3)	6	6	6	6
E. coli (isolate 4)	6	6	6	6
E. coli (isolate 5)	6	6	6	6
S. aureus (isolate 6)	6	6	6	6
Proteus spp (isolate 7)	6	12	6	14
E. coli (isolate 8)	6	6	6	6
Klebsiella spp (isolate (9)	6	6	6	7
Proteus mirabilis (isolate 10)	15	6	6	12
Pseudomonas aeruginosa (isolate11)	10	6	6	6
Shigella flexneri (isolate 12)	15	19	23	10
Pseudomonas aeruginosa (isolate 13)	10	6	6	6
E. coli (isolate 14)	15	6	6	6
Proteus spp (isolate 15)	15	17	6	8

2. Antimicrobial assay for Melia volkensii barks

***Melia volkensii* Barks**

Microorganisms	**Hexane extract**	**Methanol extract**	**Acetone extract**	**Water extract**
QC *S. aureus*	6	6	16	6
CLIN *S. aureus*	6	6	20	6
QC *E. coli*	6	6	6	6
CLIN *E. coli*	6	6	6	6
Vibrio cholerae	6	6	18	6
Salmonella typhi	6	6	6	6
Shigella dysenteriae	6	6	15	6
Enterobacter aerogenes (isolate 2)	6	6	6	8
E. coli (isolate 3)	6	6	6	6
E. coli (isolate 4)	6	6	6	6
E. coli (isolate 5)	6	6	6	6
S. aureus (isolate 6)	6	6	6	6
Proteus spp (isolate 7)	6	6	22	6
E. coli (isolate 8)	6	6	6	8
Klebsiella spp (isolate (9)	6	6	6	7
Proteus mirabilis (isolate 10)	6	6	6	8
Pseudomonas aeruginosa (isolate11)	6	6	13	6
Shigella flexneri (isolate 12)	6	6	16	6
Pseudomonas aeruginosa (isolate 13)	6	6	15	6
E. coli (isolate 14)	6	6	6	6
Proteus spp (isolate 15)	6	6	23	6

3. Antimicrobial assay for *Melia volkensii* leaves

***Melia volkensii* Leaves**

Microorganisms	**Hexane extract**	**Methanol extract**	**Acetone extract**	**Water extract**
QC *S. aureus*	6	6	6	6
CLIN *S. aureus*	6	6	6	6
QC *E. coli*	6	6	6	6
CLIN *E. coli*	6	6	6	6
Vibrio cholerae	6	6	6	6
Salmonella typhi	6	6	6	6
Shigella dysenteriae	6	6	6	6
Enterobacter aerogenes (isolate 2)	6	6	10	7
E. coli (isolate 3)	6	6	6	6
E. coli (isolate 4)	6	6	6	6
E. coli (isolate 5)	6	6	6	6
S. aureus (isolate 6)	6	6	6	7
Proteus spp (isolate 7)	6	6	6	6
E. coli (isolate 8)	6	6	6	6
Klebsiella spp (isolate (9)	6	6	6	7
Proteus mirabilis (isolate 10)	6	6	6	8
Pseudomonas aeruginosa (isolate11)	6	6	6	6
Shigella flexneri (isolate 12)	6	6	6	6
Pseudomonas aeruginosa (isolate 13)	6	6	6	6
E. coli (isolate 14)	6	6	6	6
Proteus spp (isolate 15)	6	6	6	6

4. Antimicrobial assay for Albizia coriaria barks

***Albizia coriaria* Barks**

Microorganisms	**Hexane extract**	**Methanol extract**	**Acetone extract**	**Water extract**
QC *S. aureus*	6	6	6	6
CLIN *S. aureus*	6	7	6	6
QC *E. coli*	6	7	6	6
CLIN *E. coli*	6	6	6	6
Vibrio cholerae	6	6	6	6
Salmonella typhi	6	6	6	6
Shigella dysenteriae	6	6	6	6
Enterobacter aerogenes (isolate 2)	6	6	6	7
E. coli (isolate 3)	6	6	6	6
E. coli (isolate 4)	6	10	6	6
E. coli (isolate 5)	6	7	6	6
S. aureus (isolate 6)	6	13	6	8
Proteus spp (isolate 7)	6	6	6	6
E. coli (isolate 8)	6	7	6	8
Klebsiella spp (isolate (9)	6	6	6	7
Proteus mirabilis (isolate 10)	6	6	6	8
Pseudomonas aeruginosa (isolate11)	6	6	6	6
Shigella flexneri (isolate 12)	6	10	6	6
Pseudomonas aeruginosa (isolate 13)	6	6	6	6
E. coli (isolate 14)	6	6	6	6
Proteus spp (isolate 15)	6	9	6	6

5. Antimicrobial assay for *Aloe secundiflora* leaves

Aloe Secundiflora Leaves

Microorganisms	Hexane extract	Methanol extract	Acetone extract	Water extract	
QC *S. aureus*		6	6	6	6
CLIN *S. aureus*		6	6	6	6
QC *E. coli*		6	6	6	6
CLIN *E. coli*		6	6	6	6
Vibrio cholerae		6	6	6	6
Salmonella typhi		6	6	6	6
Shigella dysenteriae		6	6	6	6
Enterobacter aerogenes (isolate 2)		6	6	6	6
E. coli (isolate 3)		6	6	6	6
E. coli (isolate 4)		6	6	6	6
E. coli (isolate 5)		6	6	6	6
S. aureus (isolate 6)		6	6	6	6
Proteus spp (isolate 7)		6	6	6	6
E. coli (isolate 8)		6	6	6	6
Klebsiella spp (isolate (9)		6	6	6	6
Proteus mirabilis (isolate 10)		6	6	6	6
Pseudomonas aeruginosa (isolate11)		6	6	6	6
Shigella flexneri (isolate 12)		6	6	6	6
Pseudomonas aeruginosa (isolate 13)		6	6	6	6
E. coli (isolate 14)		6	6	6	6
Proteus spp (isolate 15)		6	6	6	6