ANTIMICROBIAL PROPERTIES, PHYTOCHEMICAL COMPOSITION AND CYTOTOXICITY OF *Launaea cornuta*, *Maesa lanceolata* and *Bidens pilosa*

BY

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DECLARATION

DECLARATION BY THE CANDIDATE

This thesis is my original work and has not been submitted for any academic award in any institution of higher learning; and shall not be produced in part or full, or in any format without prior permission from the author and/ or University of Eldoret.

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DEDICATION

To my lovely wife Gladys, parents Mr. and Mrs. John Cheserek, siblings; Sheila, Sharon, Andrew and Arap Limo for their continued moral and material support.

ABSTRACT

Drug resistant pathogenic microorganisms have become an emerging clinical and public health challenge globally. Medicinal plants contain natural products of therapeutic value to human health due to their antimicrobial properties. *Bidens pilosa* dried leaf decoctions are used in Marakwet traditional medicine to treat skin infections and wounds. *Launaea cornuta* leaves and roots are used by the Suba people in Kenya to treat opportunistic diseases associated with HIV/AIDS. *Maesa lanceolata* is used in the Kenyan indigenous medical practice in the treatment of helminthes, bacterial and fungal infections. However, their antimicrobial properties and/or safety have not been scientifically evaluated. The main objective of the study was to determine the *in vitro* antimicrobial efficacy of *Maesa lanceolata, Bidens pilosa* and *Launaea cornuta* against pathogenic strains of *Shigella dysentriae, Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Candida glabrata, Aspergillus flavus, Trychophyton mentagrophytes and Microsporum gypseum.* The antibacterial and antifungal activity of methanol, dichloromethane and water extracts of the roots, stem and leaves of the plants against the test microorganisms was carried out using Kirby Bauer disc diffusion technique and acute toxicity evaluated using Vero E6 cell lines. Minimum inhibitory concentration was determined by broth microdilution method. The presence of phytochemicals was determined using thin layer chromatography. Data obtained from the study was analyzed using multifactorial Analysis of variance. Results showed that the four fungal pathogens were inhibited significantly by the methanolic, dichloromethane and water extracts of the plants with the exception of *Aspergillus flavus* that was not inhibited. Water extracts of the root and stem bark of *Maesa lanceolata* were significantly active with P-value of 0.0000 against *C. glabrata* ATCC 24433 strain with inhibition zone diameter and minimum inhibitory concentration values of 23.33mm, 31.25 mg/ml and 19.67mm, 125mg/ml respectively. Bacterial bioassays inhibition zone diameter and minimum inhibitory concentration values ranged between 6-21mm, 3.91-500 mg/ml respectively. The phytochemicals present in the leaves, stems and roots included alkaloids, phenols, terpenoids, anthraquinones and tannins. The selected leaves (dichloromethane and methanol) extracts and stem bark (dichloromethane and aqueous) extracts displayed cytotoxicity on Vero E6 cell lines with cytotoxicity concentration ranging between 206 - 684 µg/ml. Herbalists and the public would benefit from the information disseminated concerning the minimum inhibitory concentration to be administered to patients, which parts of the plants are most effective against infection-causing pathogenic bacteria, fungi and safety of the extracts .

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LIST OF ABBREVIATIONS

ATCC – American Type Culture Collections

CLSI - Clinical Laboratory Standard Institute

CMR – Center for Microbiology Research

CTMDR – Center for Traditional Medicine and Drug Research

DCM – Dichloromethane

DMSO – Dimethlsulphoxide

EUCAST – European Committee on Antimicrobial Susceptibility Testing

FBS – Fetal Bovine Serum

IZD – Inhibition Zone Diameter

KEMRI – Kenya Medical Research Institute

MDR – Multidrug Resistant

MHA – Mueller Hinton Agar

MIC – Minimum Inhibitory Concentration

MTT – (3-(4, 5-dim-3, 4-ethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) dye

NCCLS - National Committee for Clinical Laboratory Standards

TLC – Thin Layer Chromatography

WHO – World Health Organization

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CHAPTER ONE

INTRODUCTION

1.1 Background information

The emergence and spread of antimicrobial resistance by pathogens has greatly increased due to genetic mutations and accelerated by misuse and overuse of antimicrobials in people and animals for instance administering without professional oversight and when used as growth promoters in animals and fish (Nascimento *et al.,* 2000).

Antimicrobial-resistant microorganisms are ubiquitous in the environment (water, soil and air), people, animals and food. They are capable of spreading between people and animals and from person to person which is hastened by poor infection control, inadequate sanitary conditions and inappropriate food-handling. Classical instance of inappropriate use is presented by quinine an important antimalarial drug for almost 400 years is no longer effective in the control and management of malaria due to poor compliance with complex dosing regimens (Achan *et al*., 2011). The state of concerns has been complicated further by the emergence of HIV/AIDS, which has culminated in the recurrence of previously controlled diseases such as pneumonia, candidiasis, tuberculosis and typhoid as opportunistic infections. These contemporary dreary health tendencies consequently demand for renewed approach on prevention and treatment of infectious diseases. A diversity of flora exploited as traditional medicine has been established to treat different human diseases, which are related to microbial infections (Kokwaro, 1993).

Arokiyaraj *et al*., (2012) stated that a number of commercial drugs used in contemporary medication are obtained from plants pursuant to ethno-botanical and ethno-medical

comprehension. These medicinal plants enclose bioactive compounds capable of treating various disorders caused by microorganisms. It is hypothesized that such compounds may have developed in plants as self-defense mechanism against pests and pathogens purposely to survive and perpetuate themselves in their ecosystem (Sirikantaramas *et al*., 2014)

The United Nations World Health Organization (WHO) estimates that as many as 5.6 billion people, 80% of the world inhabitants, utilize herbal medicine for primary health care (Shen *et al.,* 2012). Medicinal plants have formed the basis of complex traditional medicine systems for thousands of years whose application have been used to treat a wide range of diseases. In the countryside, the escalating dependence on traditional medicine is largely attributed to both economic and cultural factors (Olala, 2014).

In traditional medicine, the possibility to overdose a patient due to erroneous nature of disease diagnosis and prescribed amount is of great concern not only in Kenya but also worldwide. The choice of the medicinal plants for this study was grounded on their traditional use for instance, in Marakwet community. Screening the phytochemistry of plant extracts particularly those bearing medicinal significance so as to identify phytochemical constituents responsible for bioactivity is paramount. The present study investigated the antimicrobial activity, toxicity and phytochemical composition of *Launaea cornuta, Bidens pilosa* and *Maesa lanceolata* traditionally used in Marakwet ethnomedicine.

1.2 Statement of the problem

Infectious diseases are major health concerns that adversely affect human health in Kenya and other developing countries, the resistance of bacteria and fungi to antimicrobial agents

is a world-wide medical predicament. Antimicrobial agents have reduced the threat posed by infectious diseases over a period of time since their discovery in the 1950s. However, the significant increase in the prevalence of microbial resistance to antibacterial agents such as penicillin, augmentin, kanamycin and antifungal agents at hand such as fluconazole, nystatin, ketoconazole in the recent years has greatly jeopardized these gains leading to morbidity, mortality and economic losses. This has drawn worldwide attention due to the high impact on public health.

A recently established report by the British government published in *The Standard Newspaper* dated 8th June, 2016 warns that 10 million people could die every year by 2050 from diseases caused by pan-drug resistant pathogens, so referred to as "superbugs" displaying antimicrobial resistance. Of the 10 million virtually 4.1 million will be in Africa Over time microorganisms develop resistance to antimicrobial agents due to mutations leading to the emergence of multidrug resistant (MDR) strains. Such strains are resistant to first line treatment and also the more expensive second and third-line antibiotics. The high cost of such replacement drugs and the toxicological effects are prohibitive and are out of reach for many Kenyans.

Globally, diarrheal diseases persist to be a major public health menace causing approximately 1.7 billion mortality cases annually (WHO, 2014). In Kenya, 16 % of deaths among children below five years is caused by diarrhea and is the third leading cause of outpatient cases (Njuguna and Muruka, 2011). The major causative agents of diarrheal diseases are *Salmonella typhi, Shigella, E. coli* and *Staphylococcus aureus* which is responsible for food poisoning (Mead *et al*., 1999). Therapeutic costs incurred due to pediatric diarrheal incidents have been quantified. In Kenya, the estimated average per-

episode total familial costs (direct and indirect) ranged from US$19.86 for hospitalized children (Tate *et al*., 2009).

On 27th February, 2017 W.H.O through its media center published its first ever list of antibiotic resistant "priority pathogens" that pose the greatest threat to human health. The list is categorized into three according to the urgency of need for new antibiotics as: critical, high and medium priority. The most critical group include multidrug resistant bacteria such as *Pseudomonas aeruginosa, Acetinobacter baumannii* and Enterobacteriaceae (*E. coli, Serratia, Klebsiella* and *Proteus*) are hazardous in hospitals, nursing homes and patients whose care requires devices such as ventilators and blood catheters. Methicillin-resistant *Staphylococcus aureus* is not only the major cause of nosocomial infections transmitted from patient to patient attributed to congestion in hospitals, inadequate staffing, unreliable water supply for hand washing, lack of alcohol for hand rub, isolation facilities or expertise for infection control but also food poisoning outbreaks (Forbes and Schaberg, 1983; Wertheim *et al*., 2005).

1.3 Justification

Approximately 80% of the Srural populations in Sub-Saharan Africa (SSA) rely on traditional herbal remedies for primary health care (Njiire *et al.,* 2012). In Kenya, diverse communities especially from the poor rural areas still depend on herbal remedies. Besides, many Kenyans believe in the potency of herbal medicine, even when they can access modern medicine. In some instances, others prefer to combine both herbal and conventional medicine, particularlyA if they are plagued with chronic ailments such as cancer, hypertension, infertility, HIV/AIDS and diabetes (Nagata *et al.*, 2011). Therefore,

provision of safe and effective traditional medicines could become a critical tool in increasing access to health care (WHO, 2002).

Some medicinal plants have shown the ability to overcome resistance in some organisms and this has led to researchers' investigating their mechanisms of action and isolating active compounds (Lewis and Ausubel, 2006). Development of multidrug resistant strains has been attributed to mutations and indiscriminate use of antimicrobial agents over time (Bonnet, 2004). Such strains are resistant to first line of treatments and also the more expensive second and third-line antibiotics. Herbal remedies may offer novel treatment options which elicit little transferred resistance if used in optimal concentrations. Moreover, the more efficient inhibition and lysis of bacteria resulting from the synergistic reactions of the active ingredients in plant extracts decreases the risk of progressively increasing antibiotic resistance (Miyasaki *et al.*, 2010).

On the other hand, fungal infections attributed to yeasts and dermatophytes are responsible for dermatological conditions in immune compromised individuals (Miller *et al.*, 2006).

Goldsmith and Lowel, (1983) reported that fungal infections in immunocompromised patients are ranked highest as the cause of mortality and morbidity. Antimicrobial resistance to the existing drugs is escalating healthcare costs, mounting disease severity culminating in increased morbidity and mortality. In this regard, urgent research on any potential source of novel effective antimicrobials *in situ* is called for. Natural plant products are some of such sources, which have not been utilized comprehensively.

The safety of these medicinal plants used for traditional remedies need to be investigated. Medicinal plants present new therapeutic alternatives for the treatment of infections caused by resistant microorganisms and the need to evaluate their efficacy and validation is paramount. Notwithstanding the fact that the cost of drugs is a sizable proportion of the total health expenditure in Kenya, drug related expenses account for up to 30-50% of the total cost of healthcare (WHO, 2007). The problem is compounded by the many people living in rural areas several kilometers from health centers hence not accessible to even primary health care.

People make use of traditional medicine due to its professed efficacy both in the treatment of general and specific diseases particularly chronic and devastating diseases that defy conventional pharmaceuticals (Syndara *et al*., 2005). Coupled to this, traditional medicine are popular because many people believe that it is natural and pose less adverse effects compared with some pharmaceutical based therapies (Fraenkel *et al*., 2004). This study therefore, sought to determine the antimicrobial activity, toxicity and phytochemical composition of *Launaea cornuta, Bidens pilosa* and *Maesa lanceolata* against pathogenic microorganisms particularly *Escherichia coli, Shigella dysenteriaea, Pseudomonas aeruginosa, Staphylococcus aureus, Candida glabrata, Microsporum gypseum, Trychophyton mentagrophtes, Cryptococcus neoformans* and *Aspergillus flavus.*

1.4 Objectives

1.4.1 Broad Objective

To determine the *in vitro* antimicrobial activity, toxicity and phytochemical composition of *Launaea cornuta, Maesa lanceolata* and *Bidens pilosa*.

1.4.2 Specific objectives

i) To evaluate *in vitro* antibacterial and antifungal activities of extracts of *Launaea cornuta, Maesa lanceolata* and *Bidens pilosa* against select human pathogens.

ii) To determine the phytochemical compounds present in the root, stem and leaf of *L. cornuta, M. lanceolata* and *B. pilosa*.

iii) To evaluate *in vitro* cytotoxicity of active extracts from *Launaea cornuta, Maesa lanceolata* and *Bidens pilosa* utilizing Vero E6 cell lines.

iv) To evaluate *in vivo* acute toxicity of active extracts of *L. cornuta, M. lanceolata* and *B. pilosa* by means of Swiss albino mice.

1.5 Hypotheses

i) *Launaea cornuta, Maesa lanceolata* and *Bidens pilosa* do not possess antibacterial and antifungal activities.

ii) Phytochemical compounds are not present in the root, stem and leaf of *L. cornuta, M. lanceolata* and *B. pilosa*.

iii) Active extracts from *L. cornuta, M. lanceolata* and *B. pilosa* are not cytotoxic to Vero E6 cell lines.

iv) Active extracts from *L. cornuta, M. lanceolata* and *B. pilosa* are not toxic to Swiss albino mice.

CHAPTER TWO

LITERATURE REVIEW

2.1 Use of medicinal plants

Since time immemorial, plants have served as a valuable source of natural products of therapeutic value to human health. According to World Health Organization (WHO), medicinal plants provide a variety of drugs (Arunkumar and Muthuselvan, 2009). The use of herbal medicine is popular among the majority of the people in the world especially in Asia, parts of Latin America and Africa. It is estimated that 80% of the mentioned regions use herbal medicine (Mosihuzzaman, 2012). Various plants have formed the base of traditional medicine for thousands of years with those having medicinal value applied to treat a wide range of disease categories. The first documentation on the use of medicinal herbs dates back to the 26th century BC in Mesopotamia, Egyptians and Greeks dates back to the18th century BC and the 5th century BC, respectively. In the early 11th century BC, the Chinese and Indians began to develop herbal medicine systems that continue to be practiced extensively today (Long *et al.*, 2003).

2.2 Microbial infections and their effects on human health

Microbial infections have caused serious health threats to human beings with virus, bacteria and fungi taking the lead among common microorganisms responsible for opportunistic infections particularly those associated with HIV/AIDS (Rali, 2016). In developing countries, microbial infections are commonly exhibited and attributed to factors such as poor sanitation, contaminated water and congestion in the living conditions (Worrell, 2016). Pathogenic microorganisms cause illness in several ways. Some destroy the tissue

directly. The crisis of antimicrobial resistance has been exacerbated by the emergence of HIV pandemic and other immune suppressing conditions particularly in persons suffering from cancer, stress, organ transplant and old age have led to the surfacing and resurfacing of opportunistic infections consequently leading to indiscriminate use of antibiotics (WHO, 2004). In Sub-Saharan Africa fungal infections such as cryptococcosis of the central nervous system affect one million persons annually especially human immunodeficiency virus (HIV) positive patients which accounts for 600,000 deaths annually coming fourth position amid causes of death attributed to infections (Park *et al*., 2009). In Kenya, approximately 40 % of people with HIV/AIDS develop cryptococcal meningitis (Ouma, 2001) and that the mortality rate is 10-25 % with most fatalities occurring within the first two weeks on commencement of therapy (Aberg and Powderly, 2002).

2.2.1 Fungal infections

Cryptococcosis

Cryptococcus neoformans is encapsulated yeast that is facultative intracellular pathogen indispensably an opportunistic pathogen causing cryptococcosis particularly in immunocompromised persons (Coelho, 2014). It is mainly found in the soil, dirt and bird droppings. It enters the body through inhalation into the lungs, through blood, spinal column and brain where it manifests severe disease conditions (Eisenman, 2007). Meningitis ensuing as a secondary infection particularly in AIDS patients, very often is attributed to *C. neoformans*.

Globally, it is estimated that cryptococcosis of the central nervous system affect one million persons annually particularly those that are human immunodeficiency virus (HIV)

positive and reside in sub-Saharan Africa (Park *et al*., 2009). Furthermore, it is reported that 600,000 deaths occur due to cryptococcosis among these patients yearly, an estimate that position it fourth amid causes of death attributed to infection in sub-Saharan Africa (Park *et al*., 2009). In Kenya, approximately 40% of people with HIV/AIDS develop cryptococcal meningitis and that the mortality rate is 10-25% with most fatalities occurring within the first two weeks on commencement of therapy (Aberg and Powderly, 2002).

Candidiasis

Candidiasis is a fungal infection caused by any of the *Candida* speies, in which case *Candida albicans* is the most prevalent fungal species of the human microbiota that asymptomatically colonize various areas of the body particularly the gastrointestinal and genitourinary tracts of individuals. Changes in host immunity, stress, resident microbiota among other factors culminate to *C. albicans* proliferation causing a wide range of infections: superficial mucosal to hematogenously circulated candidiasis (Nobile and Johnson, 2015). It is unique unlike other forms of candidiasis because it does not have hyphae and require 6 to 8 weeks to grow, producing minor to severe swelling of tissues and erythema.

Candida glabrata on humans is normally found on skin and mucous membrane and it can be isolated from the throat, mouth, vagina, intestine and respiratory tract. It is a normal flora in which case skin and mucous serves as barrier allowing no entry of Candida into the system. However, it can gain access into the blood through cuts and bruises on the skin or the normal flora in intestine and vagina to cause infection. This effect is compounded particularly in people with immune-compromised conditions, therefore more prevalent in people suffering from cancer, diabetes, elderly and HIV/AIDS (Fidel *et al*., 1999). It

colonizes mucosal surfaces of the oral and the vaginal cavities and the digestive tract causing candidiasis (Gupta and Kohli, 2003).

Dermatophytoses

Dermatophytoses is principally caused by three genera of fungi namely: *Trychophyton, Microsporum,* and *Epidermophyton* which causes infections in animals and humans which can be acquired through contact with infected individuals, animals or contaminated soil. Owing to their ability to obtain nutrients from keratinized material, the three genera cause infections of the skin, hair and nails. Some of these infections are known as ringworm or tinea (Viegas *et al*., 2015).

i) Microsporum gypseum

Microsporum is a genus of fungi that causes *Tinea capitis, T. corpus* and other dermatophytoses (fungal infections of the skin). The fungus can infect the hair, nails, or skin and can be passed via the infected hair or scales of the skin to another animal. All bedding materials, combs, clippers, cages or any other objects with which an infected animal comes into contact become potential sources of infection to humans (Luplertlop and Suwanmanee, 2013).

ii) Trychophyton mentagrophytes

Trychophyton mentagrophytes is a fungus that causes skin infection characterized by a number of concentric rings of overlapping scales. It may also cause athlete's foot, ring worm, jock itch and similar infections of the nail, beard and skin (Voet *et al.,* 2012).

iii) Aspergillus flavus

Aspergillus flavus is a filamentous fungus plant pathogen that produces potent carcinogenic compounds called aflatoxins. Food and livestock feed contamination by aflatoxin

produced by *A. flavus* causes a substantial crop losses annually (Nierman *et al*., 2015). Furthermore, *A. flavus* is the second top cause of aspergillosis in immunocompromised human patients (Lohmar *et al*., 2016).

2.2.2 Bacterial infections

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium found in soil, water, skin flora potentially pathogenic to animals and humans. It flourishes not only in normal atmospheres, but also in both anaerobic and microaerophilic environment (Russotto *et al*., 2015). *Pseudomonas aeruginosa* is an opportunistic pathogen whereby it affects the pulmonary tract, urinary tract, burns and wounds primarily predominantly in immune-compromised individuals (Tumbarello *et al.*, 2013; Kafle *et al*., 2016). Wang *et al*., (2015) in their research reported that not only is *P. aeruginosa* known to cause infections of burn injuries and external ear (otitis externa) but also it is the most common colonizer of medical devices like catheters.

The World Health Organization (WHO) on 27th February, 2017 issued a list of antibiotic-resistant organisms, ranking them according to ‘critical’, ‘high’ and ‘medium’ priority in terms of urgency for research of new antibiotics . There were three organisms on the critical list: *P. aeruginosa* (carbapenem resistant), *Acenitobacter baumannii* (carbapenem resistant) and Enterobacteriaceae (carbapenem-resistant, ESBL producing). The three bacteria are becoming more and more common in burns units globally as they create breeding grounds for all multi-resistant pathogens (Shokrollahi and Singleton, 2017).

Escherichia coli

This is gram-negative, rod shaped, facultative bacteria and mostly normal flora living in the intestines of people and animals causing no infection. However, some *E. coli* such as *E. coli* 0157:H7 are pathogenic causing food-borne illness including cause severe anemia, hemorrhagic diarrhea or kidney failure (Liu *et al.*, 2013). This disease is transmitted through contaminated water or food or through contact with animals or persons (Lipindu *et al*., 2014). Multidrug-resistant bacteria pose certain threats in hospitals, nursing homes and patients whose care necessitates devices such as ventilators and blood catheters. They include enterobacteriaceae (such as *E. coli*, *Klebsiella*, *Serratia*, and *Proteus*), *Acinetobacter* and *Pseudomonas* species potentially cause severe and deadly infections such as bloodstream infections and pneumonia. WHO has enlisted these pathogens as the most critical priority pathogens in terms of urgency for research of new antibiotics (Shokrollahi and Singleton, 2017).

Staphylococcus aureus

Staphylococcus aureus is facultative gram-positive coccus that is frequently found on the skin as normal flora and nasal passages (Voet *et al.,* 2012). It can cause a range of illnesses as from mild infections such as pimples to life threatening diseases such as pneumonia and is exceedingly prevalent in atopic dermatitis patients who are more susceptible to it posing a challenge to treat especially if the patients have impaired immunity (Muroi and Kibe, 2005). By definition, Methicillin-resistant *S. aureus* (MRSA) is a strain of *S. aureus* that is resistant to beta-lactam group of antibiotics which include penicillin and cephalosporin and have evolved to survive treatment with beta-lactam antibiotics posing a therapeutic

challenge particularly in hospital-associated (nosocomial) infections (Peacock and Paterson, 2015).

Shigellosis

Shigellosis is a global human health menace and epidemiological reports reveal that shigellosis is the major cause of childhood morbidity and mortality, especially in developing countries accounting for 5-8 million deaths annually (Kasper *et al*., 2005). Coupled to this, approximately 140 million people suffer from shigellosis with probable 600,000 deaths annually worldwide (Wilson *et al*., 2006). *Shigella dysentriae* invade the intestinal epithelium through M cells and proceed to spread from cell to cell causing cell lysis (Greenwood *et al.,* 2012). *Shigella* infections can be transmitted via the fecal-oral route of transmission, person-to-person contact or ingestion of contaminated food or water whose early symptom is diarrhea. There are four serogroups of *Shigella*: *Shigella flexneri, S. dysenteriaea, S. boydii*, and *S. sonnei*, all of which are pathogenic to humans (Livio *et al.,* 2014).

2.3 Medicinal plants under investigation

According to Kokwaro (1993) approximately 400 plant species with traditional therapies have been documented in Kenya. The pilot study conducted depicts that the use of traditional medicine among the Marakwet community is very eminent because besides scarcity of resources to access conventional medicine, cultural beliefs have taken their precedency.

2.3.1 *Bidens pilosa*

Bidens pilosa is an upright, perennial herb widely distributed across temperate and tropical regions bearing green opposite leaves that are serrate, lobed, or dissected. It has white or

yellow flowers and long narrow ribbed black achenes /seeds (Plate 2.1a and b). The average growth height is 60 cm and a maximum of 150 cm in favorable environments (Young *et al.*, 2010).

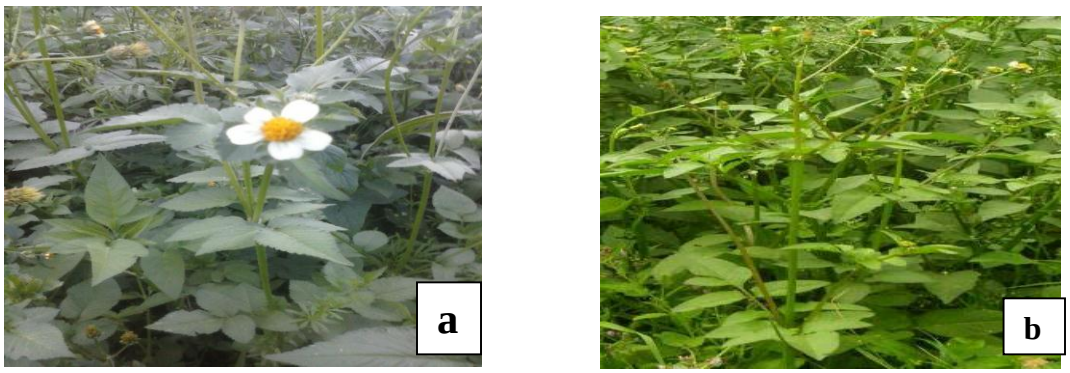


Plate 2.1: Flowered *B. pilosa* (a) and young flourishing *B. pilosa* (b). Source, author (2017)

Bidens pilosa thrives well in moderately dry soil. However, it can grow in arid and barren land from low to high elevations and is propagated by seeds (Bartolome *et al.*, 2013). Due to its fast-growing nature, in the 1970s, the Food and Agricultural Organization (FAO) actively promoted its cultivation in Africa (FAO, 1997). Conversely, due to its invasive affinity, *B. pilosa* is generally considered to be a weed.

According to Mvere (2004) *B. pilosa* is a cosmopolitan weed, originating from South America and common in all tropical and subtropical areas of the world. In Africa *B. pilosa* is recorded as a weed in many countries and it is likely to occur in all countries, including the Indian Ocean islands (Grubben, 2004).

Taxonomy of *Bidens pilosa*

Kingdom	Plantae
Subkingdom	Tracheobiota
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Asteridae
Order	Asterales
Family	Asteraceae
Genus	*Bidens*
Species	*Bidens pilosa* L.

Source: (Bairwa *et al.*, 2010)

Antimicrobial properties of *Bidens pilosa*

Their antimicrobial properties have been attributed to a group of chemicals called polyacetylenes, which includes a chemical called *phenylheptatriyne.* These phytoconstituents have displayed strong *in vitro* activity against numerous human and animal viruses, bacteria, yeast and molds in very small amounts (Bartolome *et al.,* 2013).

Bidens pilosa is used as a medicinal plant in various regions of Africa, Asia and tropical America. Their roots, leaves and seed have been reported to have antibacterial, antidysenteric, anti-inflammatory, antimicrobial, antimalarial, diuretic, hepato-protective and hypotensive activities (Lai, 2015). It is ubiquitous in Elgeyo Marakwet County and the Marakwet community traditionally uses the sap from crushed leaves to accelerate clotting of blood in fresh wounds, leaf decoction for treatment of headache and sap from the plant is used for treating ear infections. Bartolome *et al*., (2013) reported that the Giriama people from the coastal areas of Kenya use the leaf extract to treat swollen spleens in children and a mixture of the dried ground leaves, soap and hot pepper as an insecticide for the control of leaf miners and other insects.

In Uganda, five different medicinal uses are known: the sap from crushed leaves is used to speed up clotting of blood in fresh wounds; a leaf decoction is used for treating headache; sap from the plant is put in the ear to treat ear infection; a decoction of leaf powder is used to treat kidney problems; and a herbal tea made from the plant decreases flatulence (Bartolome *et al.,* 2013). Other research has focused on *B. pilosa* anti-cancerous characteristics (Lewu and Afolayan, 2009). Researchers from Taiwan have reported that a simple hot-water extract of Spanish needles could inhibit the growth of five strains of human and mouse leukemia at less than 200 μg per ml *in vitro* (Chang *et al.,* 2001; Kviecinski *et al.,* 2008; Cortés-Rojas, 2013).

2.3.2 *Launaea cornuta*

The herb is native to Africa predominantly Cameroon, Central African Republic, Ethiopia, Kenya, Malawi, Mozambique, Nigeria, Rwanda, Somalia, Sudan, Tanzania, Uganda, Zaire, Zambia, and Zimbabwe (Karau *et al.,* 2014). The plant thrives on alluvial soils in cultivated areas, on roadsides, near rivers and bush vegetation. It belongs to the family Asteraceae, and is widely distributed in almost all parts of Kenya as a troublesome weed in farms. It is an erect perennial with hollow stems up to 1.5 m high and creeping rhizomes (Plate 2.2). Morphologically, leaves form a rosette at the base, alternate on the stem, sessile, up to 25 cm long by 3 cm wide, entire or with two to three pairs of lobes acute-pointed near the base (Bakewell-Stone, 2013).

Plate 2.2: *Launaea cornuta* plant. Source, author (2017)

Medicinal uses

Launaea cornuta is utilized as a wild vegetable in African communities and source for vitamin C. In Marakwet community, a concoction from the boiled roots of *Launaea cornuta* locally called "Kipche'' is used in the treatment of throat cancer (Kigen *et al.*, 2014). The decoction is used to treat typhoid, ear pain, stomach pain, chronic joint pain, measles, gonorrhea, ascariasis, swollen testicles, warts, diabetes and in the management of breast cancer and benign prostate hyperplasia. A cold water infusion of the *L. cornuta* roots is used as a remedy for stomach ache, whilst a root-decoction combined with the leaf-sap is taken for the same trouble. According to Kareru *et al.*, (2007) a decoction from the whole *L. cornuta* is used to treat cancer and diabetes. Similarly, its leaves and roots are used by the Suba people in Kenya as antimalarial and antimicrobial particularly to treat opportunistic diseases associated with HIV/AIDS (Nagata *et al*., 2011).

Taxonomy of *Launaea cornuta*

Kingdom	Plantae
Subkingdom	Viridiphyta
Super-division	Embryophyta
Division	Tracheophyta
Class	Magnoliopsida
Order	Asterales
Family	Asteraceae
Genus	Launaea cass
Species	*L. cornuta.*

Source: (Bairwa *et al.*, 2010)

2.3.3 *Maesa lanceolata*

Maesa lanceolata Forsk is commonly known as false assegai and belongs to Myrsinaceae family. It is a sprawling shrub, 2 to 3 m tall, or a small tree with a single stem up to 9 m tall, or a rounded bushy tree with branches almost at ground level (Plate 2.3). It thrives on stream banks, cliff tops in both midland and coastal areas to about 1 500 m above sea level (Ngwenya *et al*., 2003). This plant is used in Kenya's indigenous system of medicine for the treatment of helminthes and bacterial infections (Kokwaro, 1993). Among the Marakwet community, stem bark decoction is applied to skin rashes and dermatophytic infections which heal upon consistent application (Kipkore *et al*., 2014). Manguro *et al*. (2011) investigated *M. lanceolata* leaves using aqueous methanolic extracts and purely isolated saponins, new triterpene including triterpeneglycosides. Furthermore, biological activity studies revealed MIC values of 100µg/ml for *Vibrio cholera* and 125 µg/ml for *Salmonella typhi.*

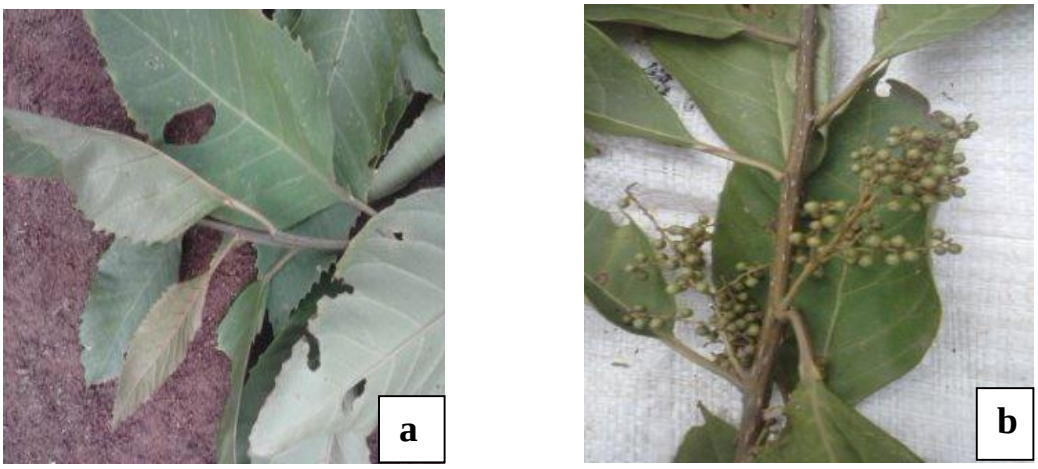


Plate 2 3: *Maesa lanceolata* young plant (a) and mature plant (b). Source, author (2017)

2.4 Herbal remedies preparation and synergism

World Health Organization (WHO) has long acknowledged the role played by the traditional medicinal plants and proposed an integrated health care system so as to ensure optimal care and avoid cases of abuse of prescribed medication. This was arrived at after analysis of the potential risks affiliated toxicity and microbial contamination of herbal extracts during preparation and administration of dosage (WHO, 2002). Oral administration or direct application on the affected area on the skin for wounds are the most common methods used (Jeruto *et al.*, 2011). According to Muroi and Kibe (2005) synergism resulting from the combination of antibiotics and extracts can eliminate the resistance offered by microorganisms to the drugs administered.

2.5 Phytochemical compounds

Phytochemicals are bioactive non-nutrient plant compounds in fruits, vegetables, grains, and other plant foods that have been linked to reducing the risk of major chronic diseases. Some are responsible for colour and other organoleptic properties, such as the coat colour of fruit and smell (Okem *et al*., 2012). There may be as many as 4,000 different phytochemicals that are responsible for antimicrobial properties of a plant (Liu, 2003).

2.5.1 Terpenoids

Terpenoids are a large and diverse class of organic compounds produced by a variety of plants particularly conifers. Preliminary studies have revealed that terpenes show antimicrobial activities attributed to their ability to disrupt membrane by the lipophilic compounds (Henry *et al*., 2015). Terpenes constitute the combination of several isoprene units while all terpenoids are composed of a group of molecules whose structure is based

on various but definite number of isoprene units (methylbuta-1, 3-diene, named hemiterpene, with 5 carbon atoms) as shown in Figure 2.1.

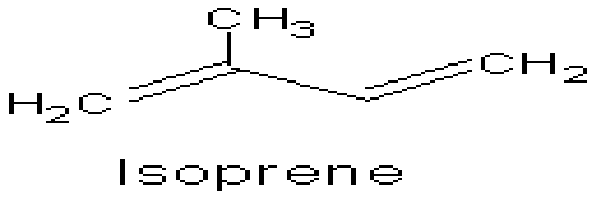


Figure 2.1: Structural formula of an isoprene

The sesquiterpenes consist of three isoprene units and have the formula $C_{15}H_{24}$ and those with the highest antibacterial activity are copaenol, cubenol and torreyol (Solis *et al.*, 2004). The presence of an -OH group which is an efficient uncoupler of the bacterial plasma membrane creates instability and breaks the membrane's phospholipid-sterol interactions (Zwenger and Basu, 2008).

2.5.2 Phenols

Phenols are chemical compounds consisting of a hydroxyl group bonded directly to an aromatic hydrocarbon group (Knob and Pilato, 2013) (Figure 2.2). Due to antioxidant properties of phenolic compounds this property has been exploited in processed foods. Some phenols such as Xylenol are used as antiseptic and formulation of disinfectants killing microorganisms by disrupting their cell membranes, precipitation of proteins and inactivation of enzymes (Franklin and Snow, 2013).

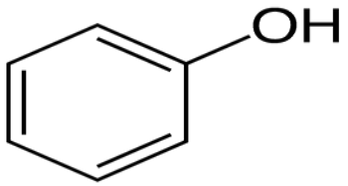


Figure 2.2: Structural formula of a phenol

2.5.3 Flavonoids

Flavonoids are water-soluble polyphenolic molecules containing 15 carbon atoms and can be visualized as two benzene rings which are joined together with a short three carbon chain (Figure 2.3). The flavonoids consist of 6 major subgroups: chalcone, flavones, flavonol, flavonone, anthocyanins and isoflavonoids. Together with carotenes, flavonoids are responsible for the colouring of fruits, vegetables and herbs (Patil, 2010). Plants synthesize them in response to microbial infection and can be effective antimicrobial substances against a wide range of microorganisms *in vitro* (Mishra *et al*., 2013).

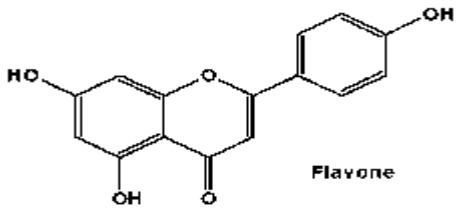


Figure 2. 3: Structural formula of flavones

2.5.4 Anthraquinones

Anthraquinones are normally found as glycosides in the living plant with several groups being distinguished based on the degree of oxidation of the nucleus and whether one or two unites make up the core of the molecule with formula $C_{14}H_8O_2$ (Figure 2.4). Anthraquinones are the main active constituents in herbs often used to relieve constipation (Portalatin and Winstead, 2012). They have a stimulating laxative effect on the large intestine. They make up the largest group of naphthaquinones, often red or purple in pigment, widely distributed in plants, especially in the *Fabaceae, Liliaceae, Polygonaceae*, and *Rhamnaceae* families (Delmulle and Demeyer, 2010).

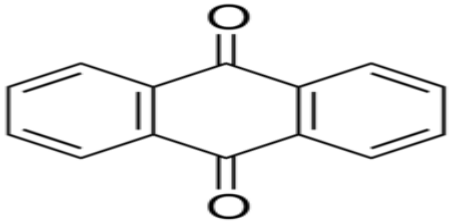


Figure 2. 4: Structural formula of anthraquinone

2.5.5 Alkaloids

Alkaloid is a chemical substance of plant origin composed of carbon, hydrogen, nitrogen, and oxygen. Many alkaloids possess potent pharmacologic effects for example cocaine, nicotine, strychnine, caffeine, morphine, atropine, methamphetamine, mescaline, ephedrine, and tryptamine. Alkaloids display good antimicrobial properties against several microorganisms (Frederich *et al.,* 2008). Roberts (2013) classified alkaloids on the basis of their chemical structure (Figure 2.5). For instance, those alkaloids that contain a ring system called indole are known as indole alkaloids. Examples include pyrrolidines, pyridines, tropanes, pyrrolizidines, isoquinolines, indoles, quinolines, the terpenoids and steroids. Alternatively, alkaloids can be classified according to the biological system in which they occur for instance, the opium alkaloids occur in the opium poppy (*Papaver somniferum*). Plant alkaloids often produce ominous effects of the remedies, however, many herbalists are of the opinion that the instant discomfort caused by the medications containing alkaloids are crucial mechanism for healing the disorder (Cheeke and Shull, 1985).

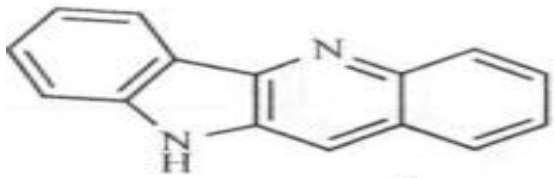


Figure 2. 5: Structure of indoloquinoline

2.5.6 Polyacetylenes

Natural polyacetylenes are compounds whose structures contain two or more triple bonds or alkynyl functional groups (Figure 2.6). Compounds containing triple bonds, like other unsaturated organic substances, are chemically and biologically active. High reactivity leading to rapid oxidation and degradation of these compounds, especially on exposure to UV light, the pH of the medium and other factors, characterizes them as relatively unstable compounds (Christensen and Brandt, 2006). Polyacetylenes have been considered undesirable in plant foods due to their toxicant properties. Some are known to be potent skin sensitizers, and neurotoxic in high concentrations, but have also been shown to have a pronounced selective cytotoxic activity against cancer cells (Kumari *et al*., 2009).

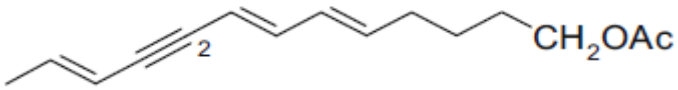


Figure 2. 6: Aliphatic polyacetylene found in species of the Asteraceae family

2.6 Mechanism of antimicrobial action

2.6.1 Antifungal mechanism and drug resistance

Cell membranes of fungi enclose sterols that antifungal agents particularly polyenes such as Amphotericin B and Nystatin bind to membrane sterols bearing a rigid hydrophobic

center and a flexible hydrophilic section. Structurally, polyenes are firmly packed rods held in rigid extension by the polyene part which interact with fungal cells to yield a membrane-polyene complex that alters the membrane permeability culminating in internal acidification of the fungus with exchange of K^+ and sugars; loss of phosphate esters, organic acids, nucleotides and ultimate leakage of fungal cell membrane and its contents (Neu and Gootz, 1996).

Faria-Ramos *et al.* (2014) reported the utilization of agricultural azoles to control plant fungal pathogens have increased the emergence of azole-resistant fungal strains. Azoles employ their action by inhibiting the C14α demethylation of lanosterol in fungi, which interferes with the synthesis of ergosterol in the cell membrane of fungi. Among the mechanisms acquired in drug resistance by fungi include mutations in azole drug target enzyme lanosterol 14α-demethylase (plays an essential role in mediating membrane permeability) and prevention of accumulation of drugs to toxic levels in the host cell (Table 2.1). For Aspergillus species some isolates have been noted to possess efflux-independent reduction in cellular drug accumulation therefore prevent drug accumulation to toxic levels in the cell (Buied *et al*., 2012).

Antifungals can be categorized into three classes grounded on their site of action:

i. Azoles- inhibit the synthesis of ergosterol (the main fungal sterol)
ii. Polyenes- interact with fungal membrane sterols physicochemically
iii. 5-fluorocytosine- inhibits macromolecular synthesis

Table 2.1: Molecular mechanisms of action of antifungal agents

Target	Chemical class	Agents
DNA and RNA synthesis	Pyrimidine	Flucytosine (5FC)
Membrane barrier function (interaction with orgesterol)	Polyenes	Amphotericin B, Nystatin
Ergosterol biosynthesis		
Squalene epoxidase	Allylamines	Naftifine, Terbinafine
	Thiocarbamate	Tolnaftate
14α-Demethylase (Cytochrome P450-14DM)	Azoles	
	Imidazoles	Bifonazole, Clotrimazole,
	Triazoles	Fluconazole, Itraconazole
Δ14-Reductase and Δ8→7 Isomerase	Morpholines	Amorolfine
Mitosis (sliding of microtubules)		Griseofulvin

Source: Bossche and Marichal (1994)

2.6.2 Antibacterial mechanism and drug resistance

Antibiotics have formed one of the most fruitful forms of therapy in medication. However, the effectiveness of antibiotics has been curtailed by the increasing emergence of antibiotic resistant pathogens. Bacteria have developed a number of mechanisms to resist antibiotics: modification of the target site so that it is not recognized by the antibiotic, chemical modification of the antibiotic, physical removal from the cell rendering it inactive or enzymatic inactivation of the antibiotic in a manner it no longer affects the microorganism (Table 2.2).

Table 2. 2: Resistance mechanisms of the different groups of antibiotics.

Antibiotic	Method of resistance
Chloramphenicol	reduced uptake into cell
Tetracycline	active efflux from the cell, ribosomal protein altered
β-lactams, Erythromycin, Lincomycin	eliminates or reduces binding of antibiotic to cell target
β-lactams, Aminoglycosides, Chloramphenicol	enzymatic cleavage or modification to inactivate antibiotic molecule i.e. altered penicillin-binding proteins
Sulfonamides, Trimethoprim	metabolic bypass of inhibited reaction
Sulfonamides, Trimethoprim	overproduction of antibiotic target (titration)

Source: Todar (2011)

2.7 Cytotoxicity assay

Cell viability is described as the quantity of healthy cells in a sample and that the proliferation of cells is a crucial pointer for understanding the mechanisms in action of particular genes, proteins and pathways involved in cell survival or death after exposing to toxic agents such as herbal extracts. Regardless of the type of cell-based assay being used, it is important to know how many viable cells are remaining at the end of the experiment. There are a number of assay methods based on numerous cell functions such as enzyme activity, cell membrane permeability, cell adherence, ATP production, co-enzyme production, and nucleotide uptake activity. Among the enzyme-based assays, the MTT assay is the best known method for determining mitochondrial dehydrogenase activities in the living cells. In the method, MTT is reduced to a purple formazan by NADH. (Mosmann, 1983).

2.8 Acute toxicity studies

Acute toxicity refers to the adverse effects of a substance such as herbal extracts that emanate either from a single exposure or from multiple exposures following oral or dermal administration in a short period of time spanning < 24 hours to 14 days on experimental animals. The assessment of the lethal dose (LD_{50}) (the dose that kills 50% of test animals population) has now been used as a major parameter in measuring acute toxicity and also as an initial procedure for general screening of chemical and pharmacological agents for toxicity. Acute toxicity study solely gives information about LD_{50}, therapeutic index and the degree of safety of a pharmacological agent (Akhila *et al*., 2007).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Collection and processing of plant samples

Bidens pilosa, Maesa lanceolata and *Launaea cornuta* were collected from Kapsowar in Elgeyo Marakwet County and identified with the help of a plant taxonomist at the University of Eldoret. Voucher specimens were deposited as follows: *Bidens pilosa* MU/0029/87, *Launaea cornuta* MU/0151/87 and *Maesa lanceolata* MU/0038/87. The plants were uprooted using a hoe and separated according to respective plant parts, dried under shade for two weeks with frequent turning until dryness and pulverized using a laboratory mill (Christy and Norris Ltd., Chelmsford, England). Packing of the powdered material was done in air tight polythene bags then stored at 4 $^{\circ}$C for subsequent procedures that followed at the Center for traditional medicine and drug research (CTMDR) KEMRI, Nairobi.

3.2 Extraction of the plant material

The solvents used for crude extraction of the fine powder were hot water (70 $^{\circ}$C), methanol and dichloromethane (Appendix I). Stock concentrations of 100 mg/ml were prepared by dissolving 1g of the crude extract into 1 ml of the respective solvents as described by Usman and Osuji (2007). The percentage yield was calculated using formula below;

$$\text{Percent yield} = \frac{\text{Weight of extract obtained}}{\text{Weight of the plant material}} \times 100$$

3.3 Aqueous extraction

Fifty grams of the crushed samples were weighed and mixed with 200 ml of distilled water. The mixture was shaken vigorously and immersed in a shaking water bath set at 70 $^{\circ}$C for

one and a half hours. After incubation in a water bath, the mixture was removed and filtered using Whatman's no. 1 filter paper on a glass funnel. The filtrate was left to cool then transferred into a round bottom flask. Using acetone and dry ice in a bucket, the filtrate in the flask was placed and rolled over the dry ice on the bowl. The frozen sample was then placed in a freeze dryer (Edwards freeze dryer Modulyo- England) so as to completely eliminate any water until it was completely dry. The dried sample was removed from the flask and stored in vials in a fridge at 4 °C for the consecutive steps.

3.4 Extraction using organic solvents

3.4.1 Extraction using methanol

Approximately 50 g of the crushed sample were placed into a 500ml conical flask and 200 ml of methanol added and shaken well. The mixture was allowed to macerate for 48 hours then filtered using Whatman's no. 1 filter paper. The filtrate was transferred into a round bottom flask and concentrated using rotary evaporator leaving a thick paste. The paste was then transferred to a sterile vial and left to dry. The crude extract was stored in a fridge at 4 °C for subsequent processes. A 100 mg sample of each extract was weighed into a sterile sample bottle and dissolved in 70% DMSO (Sigma) to make a concentration of 100 mg/ml.

3.4.2 Extraction using Dichloromethane

Approximately 50 g of the sample powder were weighed and to it was added 200ml of DCM in 500 ml conical flask. This was left to settle for 24 hours at room temperature, filtered using Whitman's no. 1 filter and the solvents removed using a rotary evaporator then air dried for three days. The contents were placed in a sterile airtight sample weighed, dissolved in DMSO (Sigma) to make a concentration of 100 mg/ml then kept in a desiccator at 4 ^{o}C in readiness for use.

3.5 Laboratory experimental design for bioassays

Experimental assemblage included selected organisms (ATCC strains and clinical isolates) and crude extracts at different concentrations. The tests were carried out to determine whether the plant extracts were active either as antifungal or antibacterial agents. Positive controls for both antifungal and antibacterial drugs included Fluconazole and Gentamicin respectively. On the other hand, the negative controls consisted of organic solvents (DMSO) and sterile distilled water necessary to ensure that the solvents used for extraction and dissolution would not have inhibitory actions. All experiments were carried out in triplicates.

3.6 Antimicrobial assays

3.6.1 Source of microorganisms

Selected ATCC and clinical bacterial and fungal pathogens were sourced from Mycology laboratory, Center for Microbiology, KEMRI. Test bacteria included the Gram positive; *Staphylococcus aureus* ATCC 25923 while Gram negative were *Escherichia coli* ATCC 25922, *Shigella dysentriae* (clinical) and *Pseudomonas aeruginosa* (clinical). The fungi examined were *Trychophyton mentagrophytes*, *Microsporum gypseum* (clinical), *Aspergillus flavus* (environmental), *Candida glabrata* and *Cryptococcus neoformans* ATCC 66031.

3.6.2 Preparation of microorganisms

Preparation for bioassays was done by sub-culturing the fungi onto Sabouraud dextrose agar (SDA) and incubating at 30 $^{\circ}$C for 72 hours respectively while the bacterial strains were sub-cultured on Mueller Hinton agar incubated at 37 $^{\circ}$C for 24 hours to obtain freshly growing strains as described by Rajakaruna and Towers (2002).

3.6.3 Preparation of McFarland standard

For purposes of standardizing the inoculum density for a susceptibility test, $BaSO_4$ turbidity standard, equivalent to a 0.5 McFarland standard were used. Precisely, 0.5 McFarland standard was prepared as described in National Committee for Clinical Laboratory Standards (NCCLS) (NCCLS, 1997). Approximately 1% V/V solution of sulfuric acid was prepared by adding 1 ml of concentrated sulfuric acid to 99 ml of water and mixed well. A 1.175% W/V solution of $BaCl_2$ was prepared by dissolving 2.35 g of $BaCl_2H_2O$ in 200 ml of distilled water. To make the turbidity standard, 0.5 ml of the $BaCl_2H_2O$ solution was added to 1% 99.5 ml Sulphuric acid and mixed well. A small volume of the turbid solutions was transferred to a screw-capped tube of the same type as used for preparing the control inocula and stored in the dark at 25 $^{\circ}$C (Andrews and BSAC, 2001).

3.7 Determination of antimicrobial activity

3.7.1 Disc diffusion assay

The antimicrobial activity of the extracts was determined by measuring the zones of inhibition using the disc diffusion method as described by (Bauer *et al.,* 1966). Test bacteria were sub-cultured and incubated for 24 hours at 37 $^{\circ}$C on nutrient agar. Molds and yeast cultures were incubated at 28 $^{\circ}$C for 96 hours and 35 $^{\circ}$C for 24 hours respectively in Potato dextrose agar supplemented with an antibiotic to obtain working cultures. From each of the cultured test microorganisms, 3 to 4 colonies were emulsified and the suspension adjusted to match 0.5 McFarland's standard so as to produce inoculated agar with 1×10^6 colony forming units/ml. The suspension was spread aseptically into Mueller Hinton agar using a sterile swabs as described by Jorgensen and Turnidge (2007).

Approximately 100 mg sample of each extract was dissolved in 1ml of each solvent to produce 100 mg/ml. Approximately 20 µl of each preparation was then measured and impregnated on to 6 mm filter paper discs prepared from Whatman No 1 that had been sterilized in an autoclave and allowed to air dry. The disks were then placed aseptically onto the inoculated plates and incubated for 24 hours at 37 ºC. Molds and yeast cultures were incubated at 28 °C for 72 hours and 35 °C for 24 hours respectively. After incubation, the inhibition zone diameters were measured in millimeters and recorded against the corresponding concentrations as described by Badria and Elgayya (2000). Discs of Gentamycin (25µg) were used as standards (positive control) for bacteria while Fluconazole discs (25 µg) were positive controls for the fungi. Discs containing 70 % DMSO were used as negative controls. Inhibition zone diameters were expressed as mean inhibition zones of the three assays. Classification of the antimicrobial activity was stipulated as ranging from little or no activity at ≤ 10 mm to very strong activity for inhibition zone diameters of ≥ 30 mm (Table 3.1) (Lee *et al*., 2004).

Table 3.1: Classification of the antimicrobial activity based on zone inhibition diameter

Activity	**Zone of inhibition (mm)**
Very strong	≥ 30
Strong	21-29
Moderate	16-20
Weak	11-15
Little or no activity	≤ 10

Lee *et al*., (2004)

3.7.2 Determination of minimum inhibitory concentration

Broth micro dilution method and disk diffusion technique were used to determine the minimum inhibitory concentration for the active crude extracts against the test microorganisms. The method recommended by the National Committee for Clinical Laboratory Standards now Clinical Laboratory Standard Institute (CLSI) (NCCLS, 2002) for antimicrobial susceptibility testing of aerobic bacteria was used. The tests were performed in 96 well-micro-titer plates whereby plant extracts were dissolved in respective solvents then transferred into micro-titer plates to make serial dilutions ranging from 10^1 - 10^{10}. The final volume in each well was 100 µl and the wells were inoculated with 5µl of microbial suspension. The yeast and bacteria were incubated at 37 $^{\circ}$C for 24 hours while molds were incubated at 28 °C for 3-7 days in ambient air. The MIC value was determined as the lowest concentration of crude extract in broth medium that inhibited visible growth of test microorganism as compared to the control where Dimethlsulphoxide (about 2 drops) dissolved in water (Motamedi *et al*., 2010). The MIC was recorded as the lowest extract concentration demonstrating no visible growth as compared to the control broth turbidity (Maregesi *et al.,* 2008). Wells that were not inoculated were set to act as controls for the experiment and the experiments were done in triplicates.

3.7.3 Data analysis for antimicrobial activity

Bioactivity for the extracts against the selected microbes was established using disk diffusion method where the zone diameter of inhibition were measured in millimeters entered into Excel spread sheet and exported to SPSS version 20 for analysis. Means were calculated, expressed as mean±standard deviation and data presented using bar graphs (Fig 4.2 to Fig 4.10).

Broth microdilution method and disk diffusion technique were used to determine the MIC for the active extracts in broth medium that inhibited viable growth of test microorganism as compared to the control broth turbidity (Table 4.2 and Table 4.3). The significant variability between and within groups: plant type, plant part, solvents and microbes at 95 % confidence interval ($p<0.05$) was determined using analysis of variance (ANOVA).

3.8 Phytochemical screening

Phytochemical screening was done on the active extracts to establish the phytocompounds present. Based on the preliminary bioassays the plant extracts exhibiting biological activity were screened for groups of chemical constituents. Thin layers chromatography (TLC) plates were developed with Ethyl acetate: petroleum spirit at the ratio of (3:7) as the solvent system for dichloromethane extracts while dichloromethane: methanol ratio (9.5: 0.5) solvent system was utilized for methanol extracts as described by Harborne (1998). After plate development a purple colour was positive for terpenoids, orange to brown colour was positive for alkaloids, yellow to brown colour was positive for flavonoids, blue colour was positive for phenolics and yellow brown was positive for anthraquinones (red to violet or green purple under ultra violet light was also positive for anthraquinones). Table 3.2 shows the TLC visualizing reagents used in this study.

Table 3.2: Thin layer chromatography visualization reagents

Compounds	Visualizing reagents
Terpenoids	Anisaldehyde
Alkaloids	Dragendorff reagents
Flavonoids	Ammonia fumes
Phenolics	Ethanolic potassium hydroxide
Anthraquinones	Methanolic potassium hydroxide (Kedde reagents)

Harborne (1984)

3.8.1 Test for tannins

One gram of the dry plant powder was stirred in 30 ml distilled water then filtered using a filter paper. A few drops of ferric chloride reagent was added into the filtrate. The formation of a blue-black or green precipitate confirmed the presence of tannins (Trease and Evans, 2002).

3.8.2 Test for flavonoids

Zero point two grams of the dry plant powder was dissolved on 4 ml ethyl acetate in a test tube vigorously shaken using a Vortex (Accumax, New Delhi, India) to ensure proper dissolution. The solution was decanted to remove plant particles and a few drops of ammonia solution were added into the filtrate. Since the liquids are immiscible the formation of an alkaline layer below the aqueous layer signify the presence of flavonoids (Dharmedra *et al*., 2012).

3.8.3 Test for saponins

Forty millimeters distilled water were boiled on a water bath for 10 minutes. Then 1 g of the dry plant powder was placed into a boiling tube and the warm water added into it and shaken for 10 minutes. The formation of thick persistent froth in the tube showed the presence of saponins (Ali *et al.*, 1990).

3.8.4 Test for terpenoids

One gram of dry plant powder was dissolved in 4 ml of chloroform in a test tube. Approximately 4 ml of concentrated Sulphuric acid was carefully added into the test tube.

The formation of a reddish colouration on the interface indicated the presence of terpenoids (Sridevi *et al*., 2012).

3.9 Cytotoxicity assay

The most active plant extracts were examined for *in vitro* cytotoxicity by the modified rapid calorimetric assay (Mossman, 1983) using Vero E6 cancer cell lines obtained from American Type Culture Collections (ATTC). The Vero cells were maintained in Eagle's Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. Approximately 2 x 10^5 cell/ml suspensions were seeded on 96-well microtiter plates and incubated in a humidified atmosphere with 5 % CO_2 at 37 $^{\circ}$C for 12 hours. Test extracts were added to the cultured cells over a concentration range of 1000µg/ml to 1.23 µg/ml. The plates were incubated at 37 $^{\circ}$C, 5% CO_2 for 48 hours followed by addition of 10µL of (3-(4, 5-dim-3,4-ethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) MTT dye into each well. The plates were then incubated for another 4 hours and subsequently the media was removed from the wells and 100µL of Dimethlsulphoxide (DMSO) were added (Mossman, 1983).

3.9.1 Data analysis on cell cytotoxicity

The criteria used to categorize the toxicity levels of the selected plant extracts depicted by the IC_{50} values were modified from those of the American National Cancer Institute (NCI) (Geran *et al*., 1972) as follows: $IC_{50} \leq 20$ mg/ml =extremely toxic, IC_{50} 21-200 mg/ml = highly toxic, $IC_{50\ 201}$-500 =moderately toxic, IC_{50} 501-999 mg/ml =lowly toxic, $IC_{50} > 1000$ mg/ml = particularly non-toxic. The plates were read (colour absorbance) using an ELISA scanning Multiwell spectrophotometer (Multiskan Ex Labssytems, Thermo Fisher

Scientific, USA) at 562 nm and 620 nm as reference. The percentage cell viability (CV) was calculated using the formula:

$$\% \text{ C V} = \frac{\text{Average abs of duplicate drug wells} - \text{Average abs of blank wells}}{\text{Average abs of control wells}} \times 100\%$$

Data was entered onto a worksheet (Microsoft Office Excel 2007) and expressed as percentage of the untreated controls. The cytotoxic concentration responsible for lysis and death of 50% of the cells was determined using Microsoft Office Excel 2007 software by linear regression analysis. The percentage cell viability of the Vero E6 cell line was determined for the extracts and presented in a line graph.

3.10 Determination of acute toxicity

One hundred and eleven Swiss male albino mice were used in the *in vivo* acute toxicity study with permission granted by the KEMRI Animal Care and Use Committee (ACUC). Healthy mice (weight 20±2g) were randomly divided into groups of three in thirty seven cages. The mice were allowed to have access to water and food, except for a short fasting period of 12 hours before oral administration of the test sample. The active extracts suspension were administered orally at logarithmic dose of 5 mg/kg, 300 mg/kg and 2000 mg/kg body weight (OECD, 2000). The general behaviour of the mice was observed continuously for one hour after the treatment and then intermittently for 4 hours, and thereafter over a period of 24 hours (Twaij *et al.,* 1983; Anaissie *et al.,* 1989). 10% Tween 80 served as the control in the experiment. The mice were observed further for up to 14 days following treatment for any sign of restlessness and the latency of death and the lethal dose was determined (Rispin *et al*., 2002). During the experiment all the dead mice were

disposed of according to KEMRI biosafety guidelines. After the experiment all the mice were sacrificed using chloroform and the carcasses incinerated.

3.10.1 Data analysis for acute toxicity

In-vivo toxicity was observed for significant differences in lethal dose resulting to death of experimental and control groups of mice at the four doses (Table 4.9). The effect of the extracts on the body weight in grams of mice following oral administration was recorded, means calculated and separated using least significant difference (LSD) (Table 4.10).

CHAPTER FOUR

RESULTS

4.1 Initial processing of samples

Out of the 200g of each powdered plant material the percentage extract yield was calculated. *Maesa lanceolata* stem bark and leaves had the highest yield of 12.1% and 5.2 % respectively on water, methanol and dichloromethane extracts (Table 4.1).

Table 4.1: Percentage yields for sequential extraction

Solvent	Plant	Part extracted	Extract weight (g)	% mean yield extract
Dichloromethane	*B. pilosa*	Leaves	6.4	3.2
		Root bark	7.6	3.8
		Stem bark	10.5	5.25
	L. cornuta	Leaves	7.8	3.9
		Root bark	5.4	2.7
		Stem bark	3.9	1.95
	M. lanceolata	Leaves	4.4	2.2
		Root bark	2.8	1.4
		Stem bark	3.6	1.8
Methanol	*B. pilosa*	Leaves	4.6	2.3
		Root bark	2.5	1.25
		Stem bark	4.8	2.4
	L. cornuta	Leaves	4.4	2.2
		Root bark	3.6	1.8
		Stem bark	4.2	2.1
	M. lanceolata	Leaves	5.3	2.65
		Root bark	10.2	5.1
		Stem bark	9.6	4.8
Aqueous	*B. pilosa*	Leaves	3.4	1.7
		Root bark	2.8	1.4
		Stem bark	3.7	1.85
	L. cornuta	Leaves	4.5	2.25
		Root bark	4.6	2.3
		Stem bark	2.6	1.3
	M. lanceolata	Leaves	9.2	4.6
		Root bark	10.4	5.2
		Stem bark	24.2	12.1

4.2 Effectiveness of the solvents used during extraction

Water, methanol and Dichloromethane solvents were used during extraction process with each displaying varying tendencies of extractability revealed by the percentage yield (Figure 4.1). When the mean percentage yields for the different solvents used in the extraction of different plant parts were compared, it was discovered *M. lanceolata* stem bark produced the highest crude extract percent yield of 12.1 % and least percent yield was observed with *L. cornuta* aqueous stem bark displaying 1.3 % yield. Aqueous stem extract produced the highest yield of 5.08 % followed by methanolic stem bark and Dichloromethane leaf extracts both yielding of 3.1 % (Table 4.1). Lowest yields were observed with methanolic leaf extracts with mean percentage yield of 2.38%.

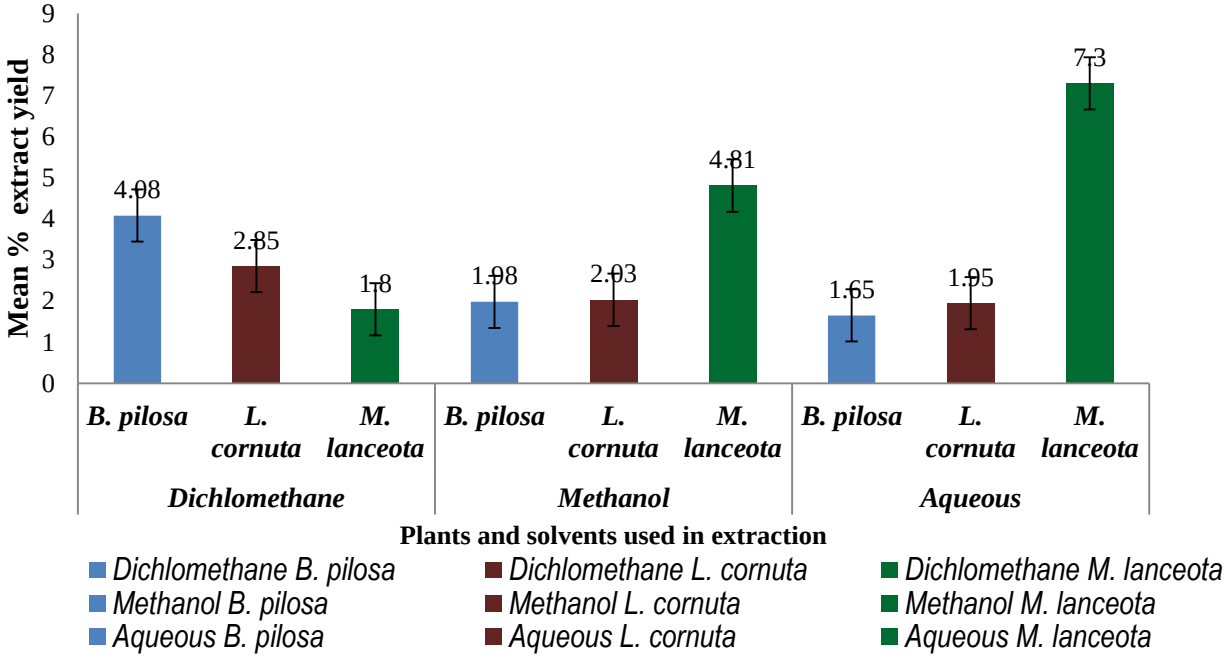


Figure 4.1: Mean % yield of different extracts per plant type selected

Aqueous and methanolic extracts of *M. lanceolata* registered the highest mean percentage extract yield of 7.3% and 4.81% respectively. On the other hand, aqueous extracts of *B. pilosa* gave the least mean percentage extract yield of 1.65%. In general, the three solvents

(Dichloromethane, water and methanol) mean percent extract yield ranged 1.60 % to 2.90 %. The three plant types and the solvents (DCM, methanol and water) used in the extraction process had no significant effect on the percentage yield with P-value of 0.420 and 0.899 respectively at 95% confidence interval.

4.3 Evaluation of the antimicrobial activity

4.3.1 Inhibition zone diameters

Initial screening test was done to establish the bioactivity of the extracts using disk diffusion method as shown in plates 4.1 and 4.2.

Key: 1- Water disc, 2-Fluconazole disc, 3- Test extract, 4- Microbe under test

Plate 4.1: *Antimicrobial activity of* M. lanceolata *extracts showing inhibition zone diameter* against C. glabrata *(a) and* S. dysenteriaea *(b)*. Source, author (2017)

4.3.2 Antibacterial bioassay

The diameters of the zone of inhibition for triplicate tests were measured (Appendix 2). The antibacterial activity of the three plant extracts were tested against four bacteria species namely; *Staphylococcus aureus* ATCC 25923, *E. coli* ATCC 25922, *Pseudomonas aeruginosa*, *Shigella dysentriae* at the Mycology and Opportunistic Infections laboratory, Center for Microbiology, KEMRI - Nairobi. The antimicrobial activity of *B. pilosa, L. cornuta* and *M. lanceolata* against *Staphylococcus aureus, Shigella dysenteriaea, P. aeruginosa* and *E. coli* are given in Figures 4.2, 4.3, 4.4 and 4.5 respectively.

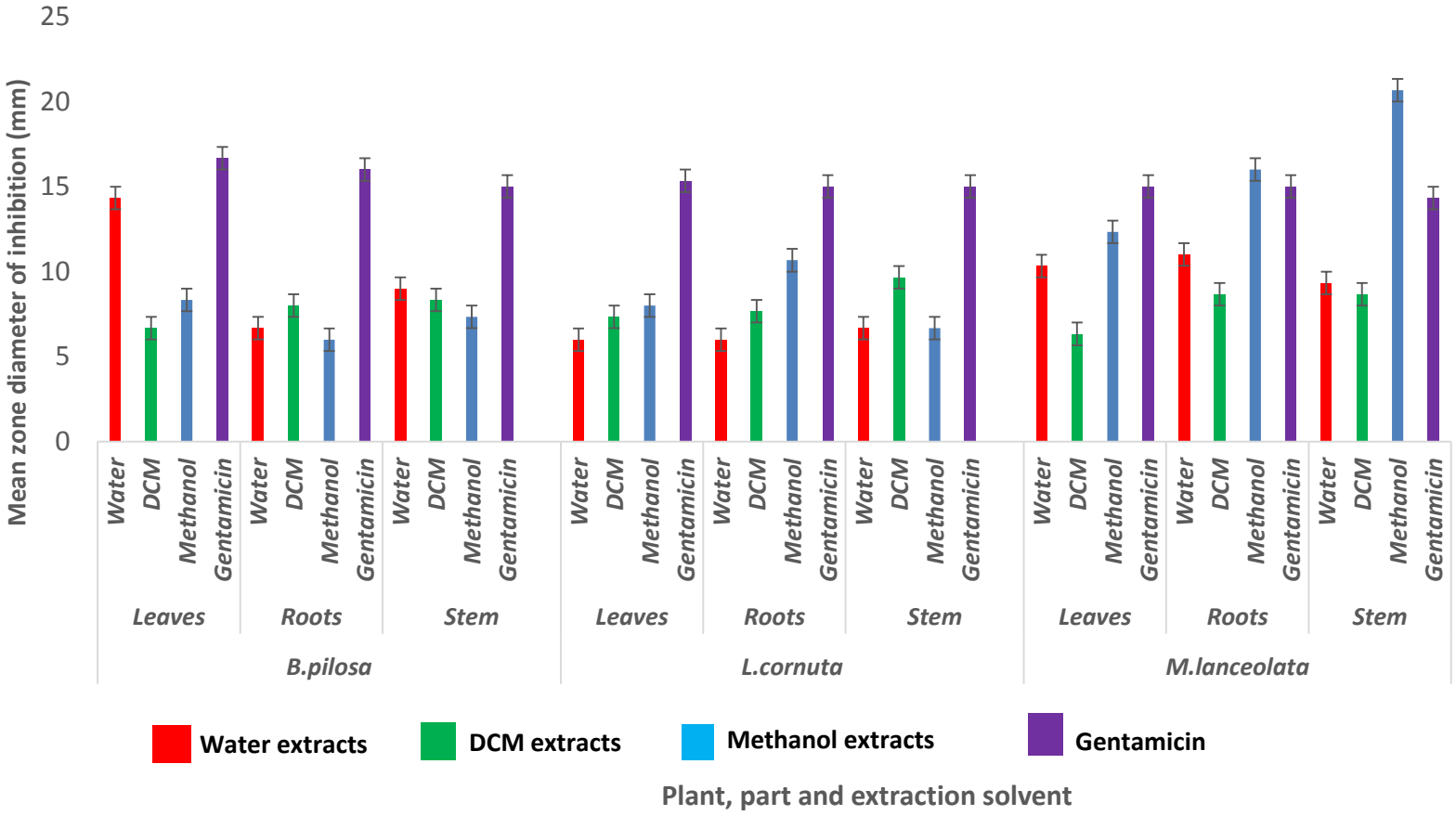


Figure 4.2: Activity of *B. pilosa, L.* cornuta and *M. lanceolata* against *Staphylococcus aureus*

Maesa lanceolata stem bark methanolic extracts displayed the largest zone diameter of inhibition depicting higher antimicrobial activity with zone diameter inhibition of 21 mm while gentamicin had a zone diameter inhibition of 14.3mm against *S. aureus* ATCC 25923. In

most cases *L. cornuta* root, stem and leave extracts were weak showing very little or no activity with zone diameter inhibition of less than 10 mm against S. aureus. *B. pilosa* aqueous leave extracts had moderate activity against *S. aureus* with IZD of 14.33 mm while gentamicin had 16.7 mm (Fig. 4.2).

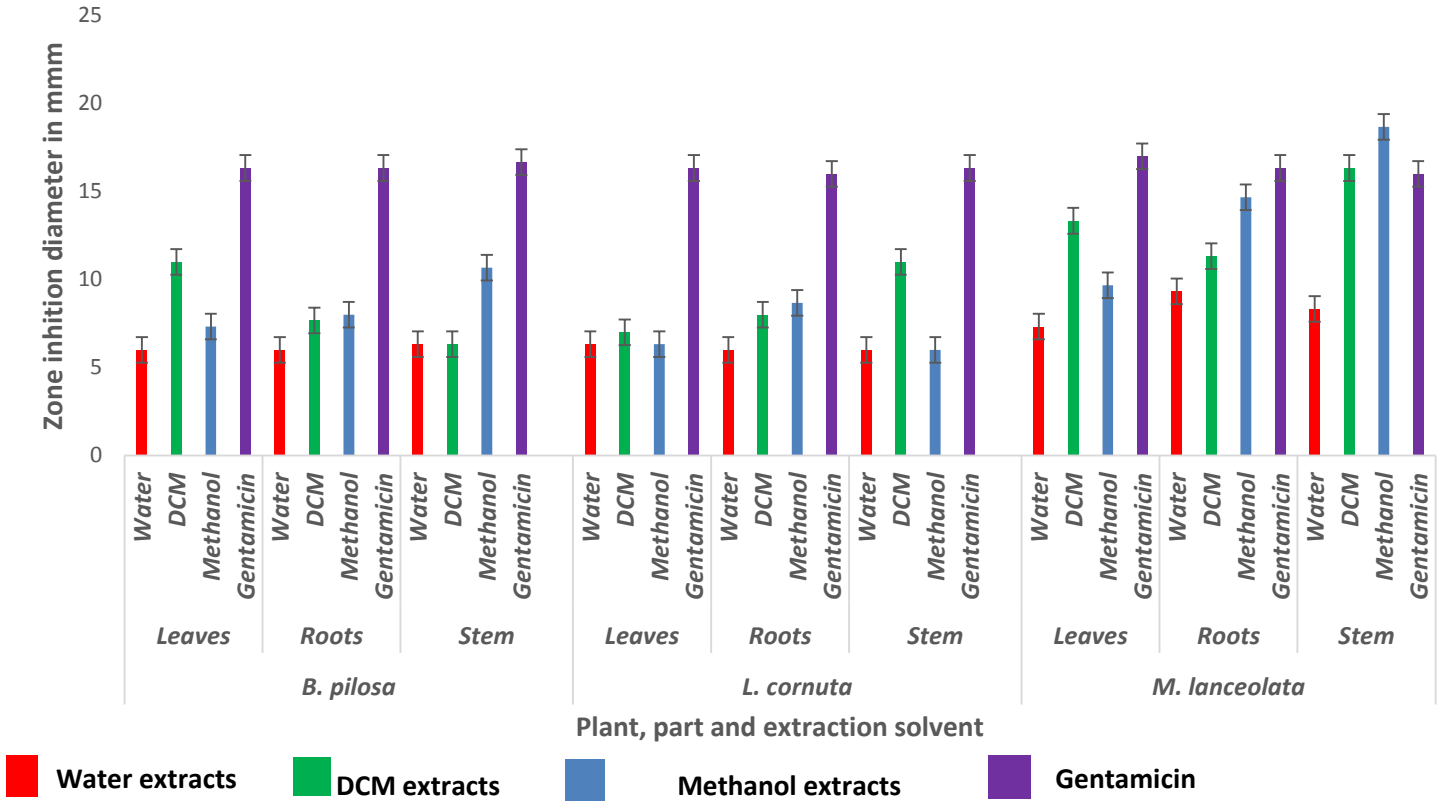


Figure 4.3: Activity of *B. pilosa, L. cornuta* and *M. lanceolata* against *Shigella dysenteriaea.*

Very little or no activity was observed with *B. pilosa* and *L. cornuta* against *S. dysenteriaea* bearing an IZD less than 10 mm as compared with gentamicin with IZD of 16 mm (Figure 4.3). For *M. lanceolata* methanol and DCM stem bark extracts showed a stronger activity with IZD of 18.7 mm and 16.33 mm respectively while gentamicin had an IZD of 16.3 mm.

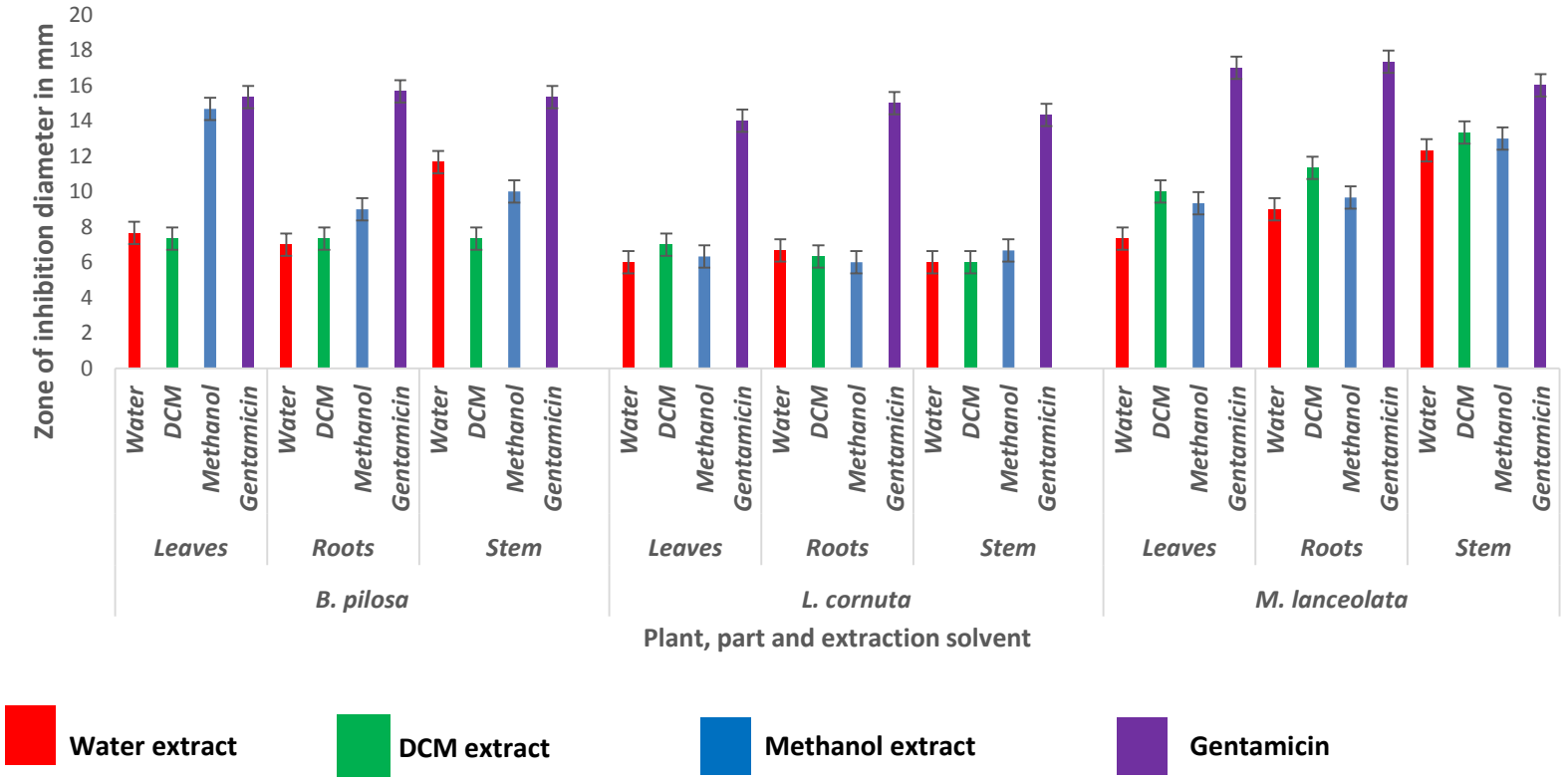


Figure 4.4: Activity of *B. pilosa, L. cornuta* and *M. lanceolata* against *Pseudomonas aeruginosa*.

Moderate activity was noted with *B. pilosa* methanol leave extracts exhibiting an IZD of 15.0 mm while gentamicin had IZD of 15.33 mm against *P. aeruginosa*. *L. cornuta* extracts displayed no activity against *P. aeruginosa.* On the other hand, *M. lanceolata* extracts were generally weak against *P. aeruginosa* evident by the IZD between 9.0 to 13.0 mm (Fig. 4.4).

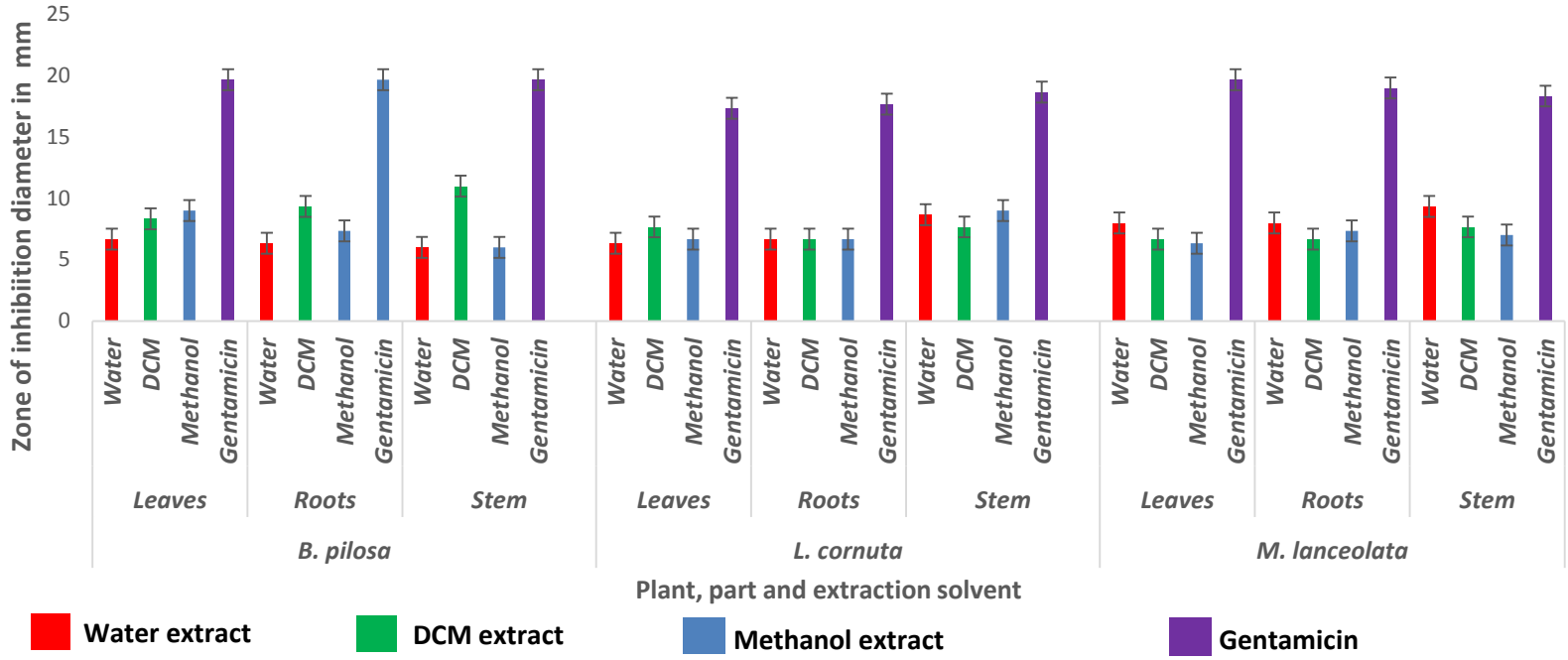


Figure 4.5: Activity of *B. pilosa, L. cornuta* and *M. lanceolata* against *Escherichia coli*

Bidens pilosa extracts exhibited weaker activity against *E. coli* evident by IZD < 9 mm as compared with gentamicin bearing IZD of 20 mm. Very little/no activity was seen with *L. cornuta* extracts against *E. coli* displayed by IZD less than 9 mm in comparison with IZD of 18.0 mm. Similar observations were made for the case of *M. lanceolata (Fig.4.5).*

4.3.3 Antifungal bioassay

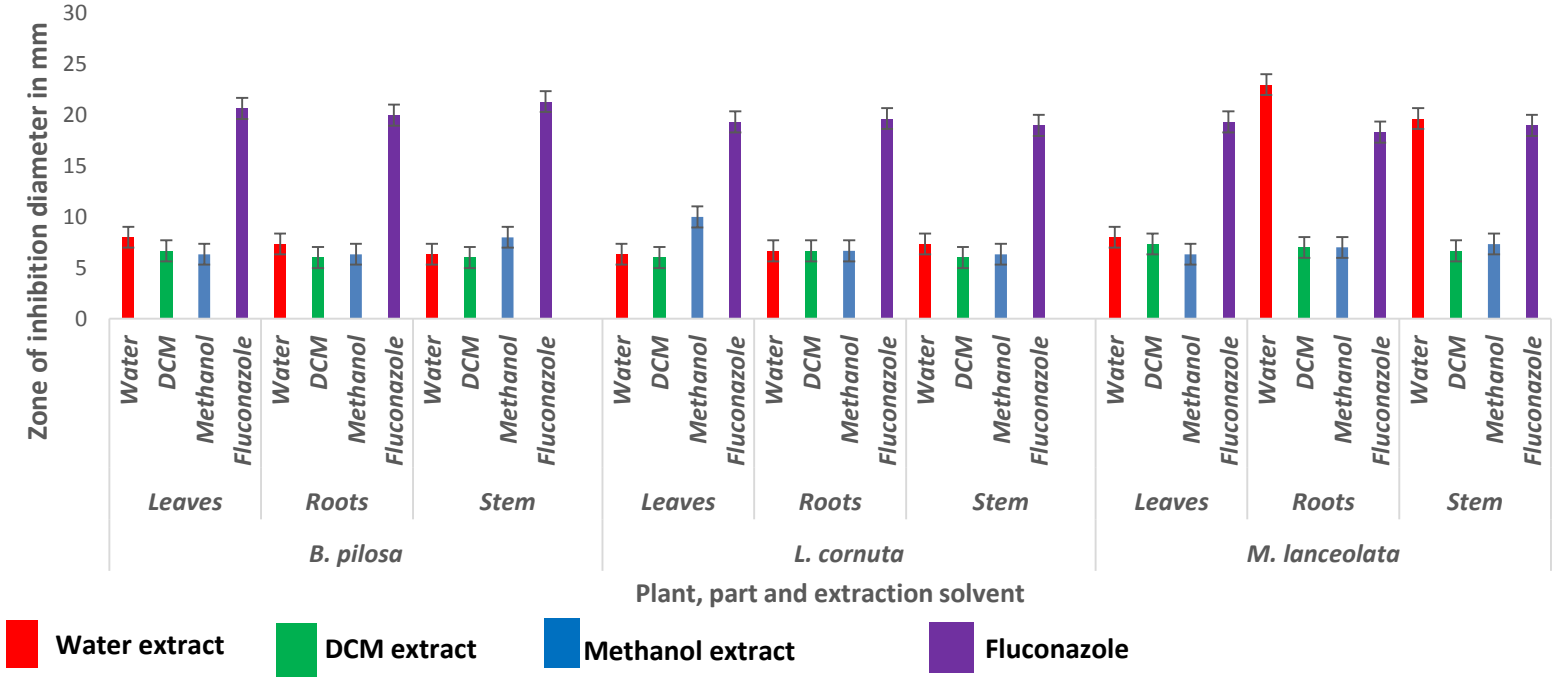


Figure 4.6: Activity of *B. pilosa, L. cornuta* and *M. lanceolata* against *Candida glabrata*

The activity of *B. pilosa, L. cornuta* and *M. lanceolata* against selected *Candida glabrata, Microsporum gypseum, Trychophyton mentagrophtes, Cryptococcus neoformans* and *Aspergillus flavus.*is seen (Fig. 4.6 - 4.10).

Generally, *B. pilosa* and *L. cornuta* extracts of water, DCM and methanol showed very little activity against all the selected fungi with IZD ranging between 6.0 mm to 10.0 mm. However, the roots and stem bark aqueous extracts of *M. lanceolata* were strongly active against *Candida glabrata* with zone inhibition diameters of 23.0 mm and 20.0 mm respectively

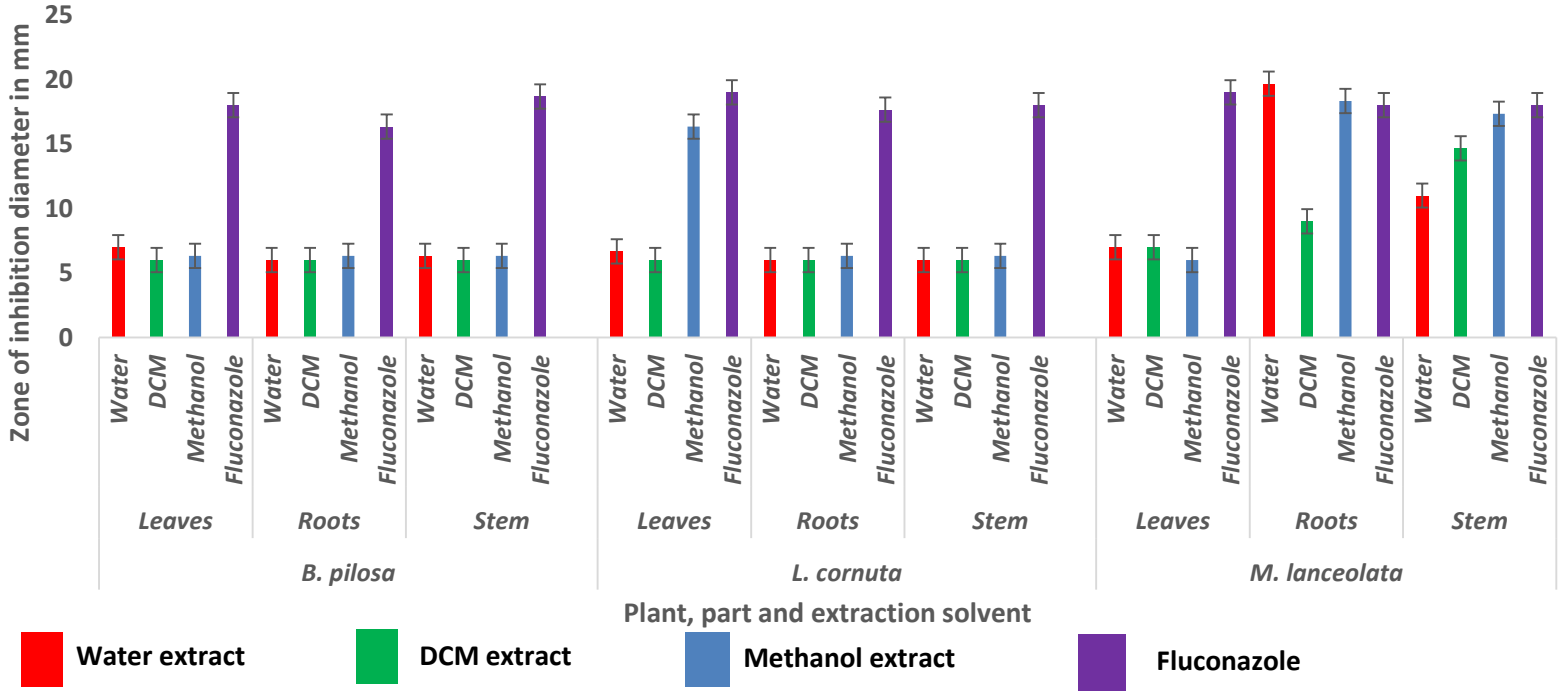


Figure 4.7: Activity of *B. pilosa, L. cornuta* and *M. lanceolata* against *Cryptococcus neoformans*

Bidens pilosa and *L. cornuta* extracts derived from their different plant parts (leave, root and stem bark) were inactive against; *Candida glabrata. Cryptococcus neoformans, Microsporum gypseum, T. mentagrophytes* exhibiting IZD ≤ 7 mm except methanol *L. cornuta* leave extracts that had a IZD of 16.33 mm. *Cryptococcus neoformans* was very susceptible to *M. lanceolata* aqueous and methanolic root extracts both displaying a zone of inhibition diameter of 20.0 mm and 19.0 mm respectively. Leave extracts of *M. lanceolata* showed zone inhibition diameter (IZD) ranging from 7 mm to 10 mm.

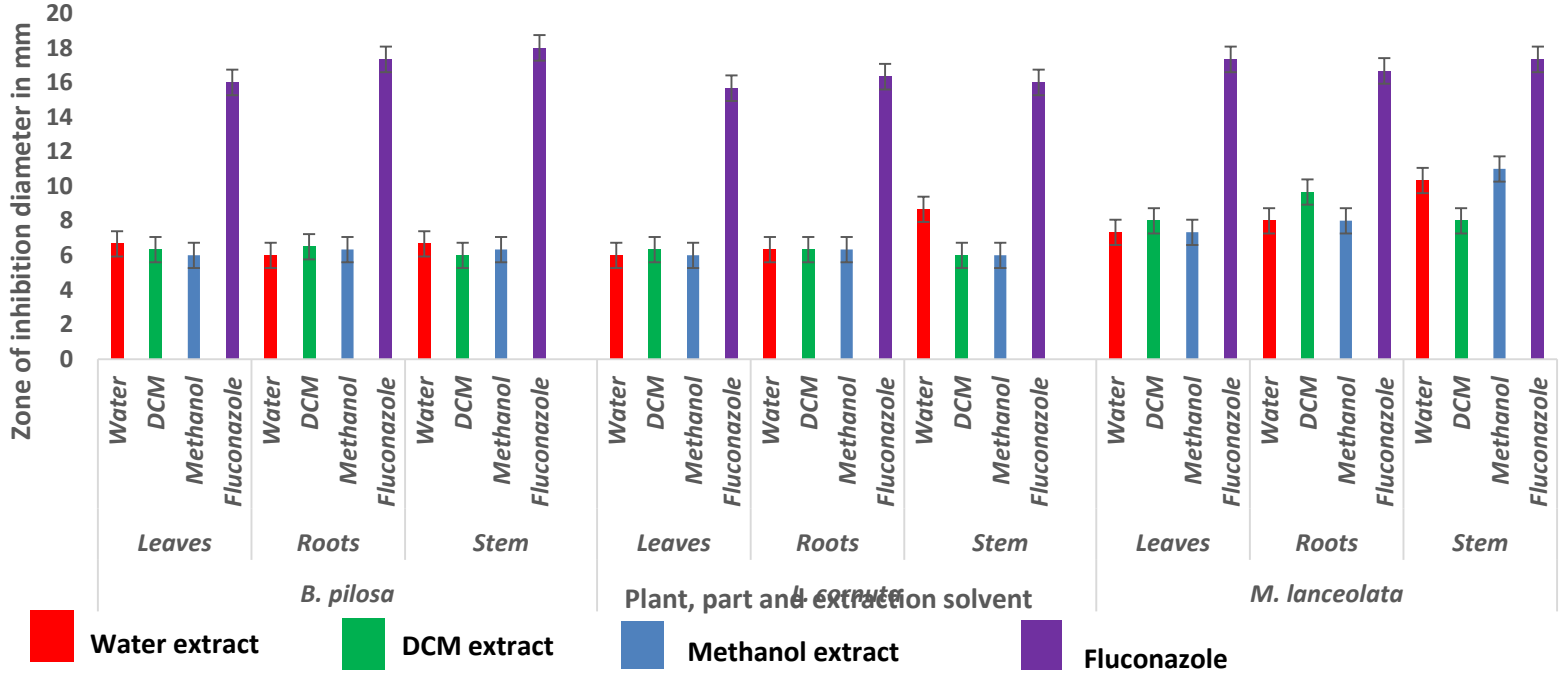


Figure 4.8: Activity of *B. pilosa, L. cornuta* and *M. lanceolata* against *Microsporum gypseum*

All extracts of *B. pilosa*, *L. cornuta* and *M. lanceolata* were weakly active against *M. gypseum* with all exhibiting IZD < 11.0 mm. Fluconazole showed an IZD range between 15.0 mm to 17.5 mm (Fig. 4.8).

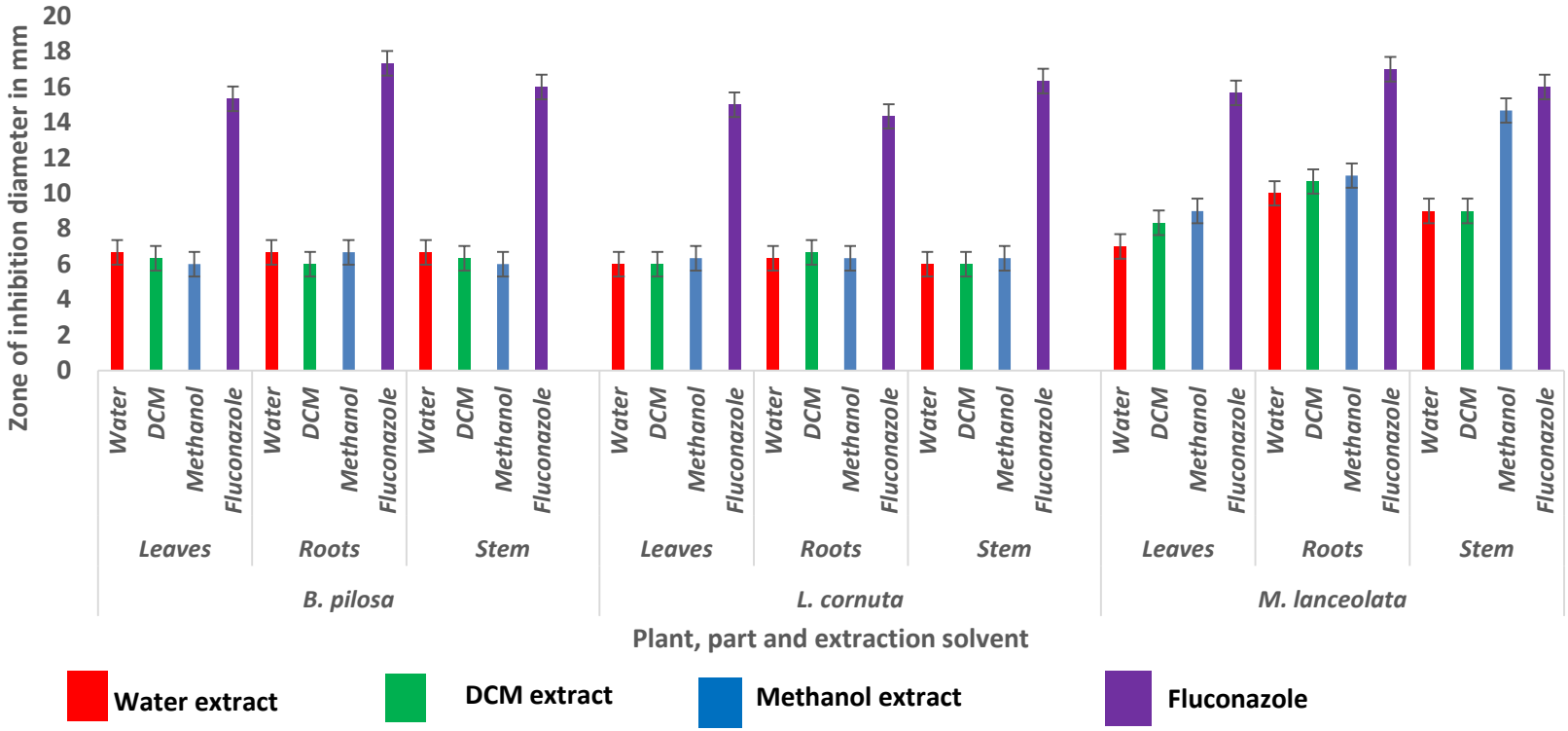


Figure 4.9: **Activity of *B. pilosa, L. cornuta* and *M. lanceolata* against *Trychophyton mentagrophytes.***

Extracts of *B. pilosa*, *L. cornuta* and *M. lanceolata* had weak activity against *T. mentagrophytes* with all exhibiting IZD < 11.0 mm. Fluconazole showed an IZD range between 15.0 mm to 17.0 mm (Fig. 4.9).

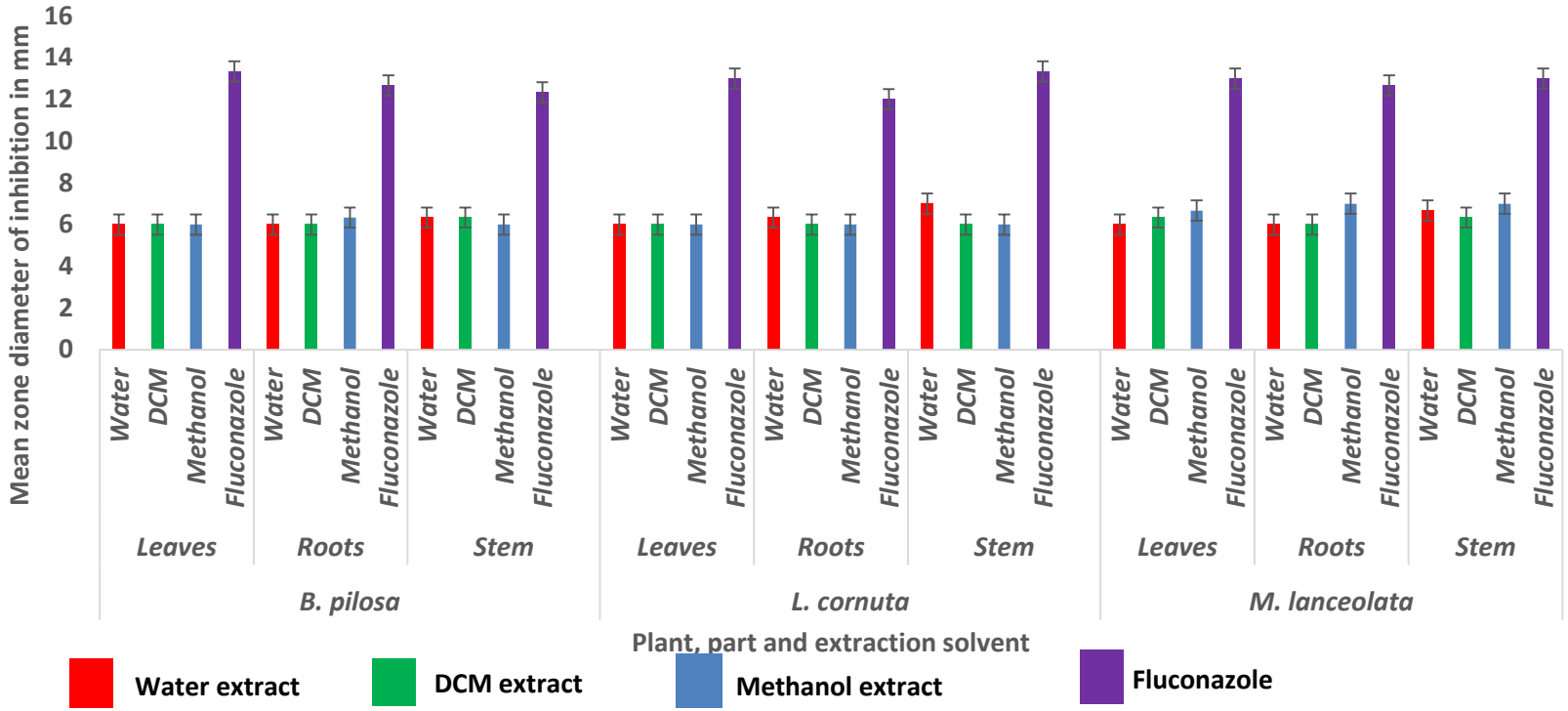


Figure 4.10: Activity of *B. pilosa, L. cornuta* and *M. lanceolata* against *Aspergillus flavus*

All extracts of *B. pilosa*, *L. cornuta* and *M. lanceolata* were totally inactive against *A. flavus* with all exhibiting IZD of 6.0 mm. Fluconazole showed an IZD range between 12.0 mm to 13.0 mm (Fig. 4.10).

4.4 Evaluation of the minimum inhibitory concentration

Plant extracts that demonstrated a significant activity were further subjected to bioassay to establish their minimum inhibitory concentration which is considered as the as the lowest concentration that produced a visible zone of inhibition. MICs were tabulated for antibacterial (Table 4.3) and antifungal (Table 4.2) isolates.

Table 4.3: MICs of plants' crude extracts for antifungal assays

Plant	Plant part	Solvent	Test organism	MIC (mg/ml)
Standard	*Fluconazole			
M. lanceolata	Roots	Aqueous	*C. glabrata*	31.25
M. lanceolata	Stem bark	Aqueous	*C. glabrata*	125
M. lanceolata	Roots	DCM	*M. gypseum*	125
M. lanceolata	Leaves	DCM	*T. mentagrophtes*	250
M. lanceolata	Roots	DCM	*T. mentagrophtes*	62.5
M. lanceolata	Stem bark	DCM	*T. mentagrophtes*	250
M. lanceolata	Roots	Methanol	*M. gypseum*	125
M. lanceolata	Stem bark	Methanol	*M. gypseum*	62.5
M. lanceolata	Leaves	Methanol	*T. mentagrophtes*	125
M. lanceolata	Roots	Methanol	*T. mentagrophtes*	62.5
M. lanceolata	Stem bark	Methanol	*T. mentagrophtes*	125
M. lanceolata	Stem bark	Aqueous	*M. gypseum*	31.25
M. lanceolata	Roots	Aqueous	*T. mentagrophtes*	62.5
M. lanceolata	Stem bark	Aqueous	*T. mentagrophtes*	62.5

***MIC for the standard drugs were determined and interpreted according to EUCAST procedures and quality control (Matuschek *et al.*, 2014).**

Maesa lanceolata stem, roots and leaves derived from DCM, water and methanol showed most activity against the fungal isolates relative to the *L. cornuta* and *B. pilosa* extracts that displayed very weak activities against the selected fungi. The lowest concentrations that

produced visible zones of inhibition for all extracts tested ranged between 250 to 31.25 mg/ml for antifungal assays. *M. lanceolata;* aqueous stem bark and aqueous root extracts were the most active against *M. gypseum* and *C. glabrata* respectively with a MIC of 31.25 mg/ml respectively.

Table 4. 2: MICs of plants' crude extracts for antibacterial assays

Plant	**Plant part**	**Sample (solvent)**	**Test organism**	**MIC (mg/ml)**
Standard	*Gentamicin			
L. cornuta	Stem bark	DCM	*S. aureus*	500
L. cornuta	Roots	DCM	*S. aureus*	125
L. cornuta	Stem bark	DCM	*S. dysenteriaea*	62.5
B. pilosa	Roots	DCM	*E. coli*	500
B. pilosa	Leaves	DCM	*S. dysenteriaea*	31.25
B. pilosa	Stem bark	Methanol	*S. dysenteriaea*	62.5
M. lanceolata	Stem bark	DCM	*S. aureus*	15.63
M. lanceolata	Leaves	Methanol	*S. aureus*	125
M. lanceolata	Roots	Methanol	*S. aureus*	7.81
M. lanceolata	Stem bark	Methanol	*S. aureus*	3.91
M. lanceolata	Leaves	Aqueous	*S. aureus*	125
M. lanceolata	Roots	Aqueous	*S. aureus*	62.5
M. lanceolata	Stem bark	Aqueous	*S. aureus*	31.25
M. lanceolata	Roots	DCM	*C.neoformans*	125
M. lanceolata	Stem bark	DCM	*C.neoformans*	62.5
M. lanceolata	Roots	Methanol	*C.neoformans*	15.63
M. lanceolata	Stem bark	Methanol	*C.neoformans*	125
M. lanceolata	Roots	Aqueous	*C.neoformans*	15.63
M. lanceolata	Stem bark	Aqueous	*C.neoformans*	62.5
M. lanceolata	Stem bark	DCM	*E. coli*	250
M. lanceolata	Roots	DCM	*P. aeruginosa*	31.25
M. lanceolata	Stem bark	DCM	*P. aeruginosa*	31.25
M. lanceolata	Roots	Methanol	*P. aeruginosa*	62.5
M. lanceolata	Stem bark	Methanol	*P. aeruginosa*	31.25
M. lanceolata	Stem bark	Aqueous	*P. aeruginosa*	31.25
M. lanceolata	Leaves	DCM	*S. dysenteriaea*	15.63
M. lanceolata	Roots	DCM	*S. dysenteriaea*	31.25
M. lanceolata	Stem bark	DCM	*S. dysenteriaea*	15.63
M. lanceolata	Leaves	Methanol	*S. dysenteriaea*	125
M. lanceolata	Roots	Methanol	*S. dysenteriaea*	62.5
M. lanceolata	Stem bark	Methanol	*S. dysenteriaea*	15.63
M. lanceolata	Roots	Aqueous	*S. dysenteriaea*	31.25

The lowest concentrations that produced visible zones of inhibition for all extracts tested ranged between 500 to 3.91 mg/ml (Table 4.3). *M. lanceolata* stem bark extracts was the most active with a MIC of 3.91 mg/ml against *S. aureus* bacteria and the weakest activity was observed with *L. cornuta* DCM stem bark extract against *S. aureus* featuring a MIC value of 500 mg/ml. Generally, *M. lanceolata* proved to show more activity against selected bacteria relative to the other two test medicinal plants. The MIC values for *M. lanceolata* ranged from 250 mg/ml to 3.91 mg/ml against the bacterial isolates.

4.5 Efficacy of selected plant parts on selected pathogens

The tested plant extracts displayed a significant effect in inhibiting the growth of fungi and bacteria $p< 0.05$ via disc diffusion technique. The zone diameter of inhibition varied significantly ($P < 0.05$) depending on the type of plant, plant part, microorganism and the solvent used during the extraction process (Table 4.4). The interactions between microorganism and plant type (microorganism × plant type), microorganism and plant part (microorganism × plant part), microorganism and solvent (microorganism × solvent), plant part and solvent (plant part × solvent), plant type and plant part (plant type × plant part), plant type and solvent (plant type × solvent) had a significant effect on the zone of inhibition at $p < 0.05$ (Table 4.4). Similarly, plant type, microorganism and solvent; plant type, microorganism and plant part; plant type, plant part and solvent; microorganisms, plant part and solvent interactions had a significant effect on the zone inhibition diameter at 95% confidence interval. The p-values for all the variables ranged 0.00000 to 0.0160; ($p<0.05$), implying that they are statistically significant and that the plant extracts under investigation can be used as antimicrobial agent (Table 4.4.and 4.5).

Table 4.2: Interaction between plant type, solvents and bacterial isolates on the IZD

Source of variation	F ratio	p value	Effect
Plant type	164.94	0.0000	S
Bacteria	3.49	0.0158	S
Plant part	1428.74	0.0000	S
Solvent	26.31	0.0000	S
Plant type × bacteria	23.63	0.0000	S
Plant type × plant part	42.01	0.0000	S
Plant type × solvent	5.86	0.0001	S
Bacteria × plant part	26.23	0.0000	S
Bacteria × solvent	12.02	0.0000	S
Plant part × solvent	8.11	0.0000	S
Plant type × bacteria × plant part	6.75	0.0000	S
Plant type × bacteria × solvent	15.1	0.0000	S
Plant type × plant part × solvent	5.24	0.0000	S
Bacteria × plant part × solvent	7.81	0.0000	S

S: Significant at p<0.05

Table 4.3: Interaction between plant type, solvents and fungi isolates on the IZD

Source of variation	F ratio	p value	Effect
Fungi	33.57	0.0000	S
Plant part	40.95	0.0000	S
Plant type	302.88	0.0000	S
Solvent	26.38	0.0000	S
Fungi × Plant part	6.2	0.0000	S
Fungi × Plant type	27.64	0.0000	S
Fungi × Solvent	17.35	0.0000	S
Plant type × Plant part	42.65	0.0000	S
Plant part × Solvent	6.21	0.0001	S
Plant type × Solvent	9.7	0.0000	S
Fungi × plant part × plant type	8.84	0.0000	S
Fungi × plant part × solvent	6.45	0.0000	S
Fungi × plant type × solvent	17.35	0.0000	S
Plant part × plant type × solvent	8.71	0.0000	S

S: Significant at p<0.05

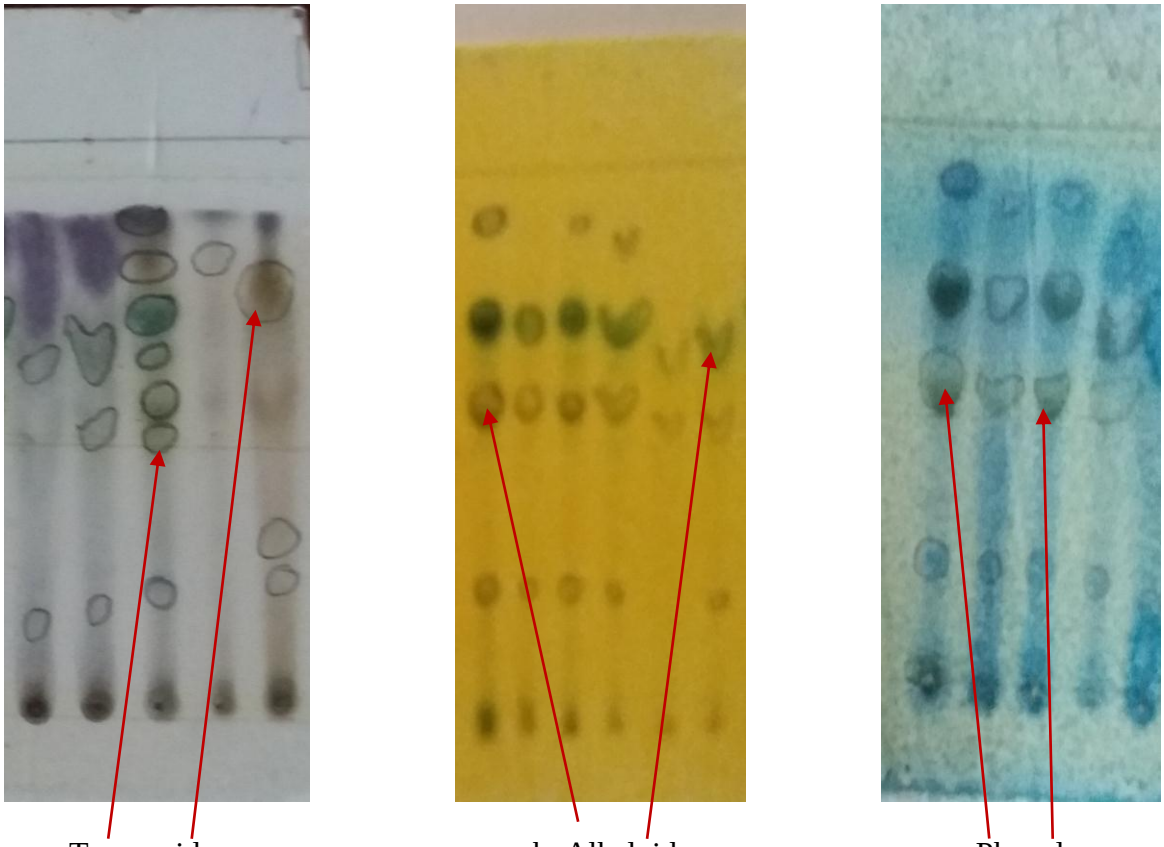
4.6 Phytochemical evaluation

4.6.1 Preliminary screening of phytochemical constituents

In the study; DCM, methanol and aqueous extracts of *B. pilosa*, *M. lanceolata* and *L. cornuta* were subjected to qualitative chemical tests using standard procedures as described by Trease and Evans (2002) to identify diverse phytoconstituents present therein.

4.6.2 Thin layer chromatography

Thin layer chromatography was used to analyze the phytochemicals of the plant extracts using aluminum-backed TLC (Merck, Silica gel 60 F254). Plates were spotted with 50 mg/ml of DCM and methanol extracts (Fig. 4.11). Two solvent systems were used: ethyl acetate/petroleum ether (3:7) for DCM extracts and DCM/methanol (9.5:0.1) for methanolic extracts at 100 mg/ml. Bands which were invisible on TLC plates in daylight were viewed with the aid of U.V. light at 254 nm and 365 nm. TLC profiling of the various plant extracts using the chosen solvent systems authenticates the presence of diverse group of phytochemicals. TLC spray reagents used for screening the phytochemicals established that phenols, tannins and saponins were contained in all the studied medicinal plants extracted from DCM and methanol solvents. Flavonoids, alkaloids, anthraquinones and terpenoids were revealed to be present in the roots, stem bark and the leaves of the plants tested (Table 4.6).



a. Terpenoids b. Alkaloids c. Phenols

Key

- a) Purple spots for terpenoids
- b) Orange spots on yellow background for alkaloids
- c) Blue colour for Phenolic compounds

Figure 4.11: Thin Layer Chromatographic plate positive for a) terpenoids derived from methanol *B. pilosa* roots, b) alkaloids from *M. lanceolata* aqueous stem bark extracts and c) phenolic compounds derived from *L. cornuta* DCM root extracts. Source, author (2017)

Phytochemicals present in the methanol, DCM and water extracts included tannins, flavonoids, phenols, saponins, terpenoids, alkaloids and anthraquinones to varying quantities in the three plants. Alkaloids were moderately present in *B. pilosa* and *L. cornuta* derived from all solvents. However, alkaloids were alkaloids were absent in DCM extracts of all plant parts of *B. pilosa* and *L. cornuta* (Table 4.6).

Table 4.4: Phytochemical profile of the plant extracts

	Solvent	Part	Tannins	Flavonoids	Phenols	Saponins	Terpenoids	Alkaloids	Anthraquinones
B. pilosa	Water	L	++	+++	+++	+++	+++	+++	+
		R	+++	+++	+++	+++	+++	+	++
		S.B	+++	++	+++	+++	+++	++	+++
	DCM	L	++	+++	+++	++	+++	-	+++
		R	++	++	+++	+++	+++	-	++
		S.B.	+++	++	+++	+++	+++	-	++
	Methanol	L	+++	+++	+++	++	+++	+++	++
		R	++	++	+++	++	+++	++	++
		S.B.	+++	+	+++	++	+++	+	++
L. cornuta	Water	L	++	-	+++	+++	+++	++	++
		R	+++	-	+++	+++	+++	+	+++
		S.B	+++	-	+++	+++	++	++	++
	DCM	L	++	++	+++	+++	+++	-	++
		R	+++	+	+++	+++	+++	-	+
		S.B.	++	+	+++	++	+++	-	+
	Methanol	L	++	-	++	+	++	-	+
		R	+	-	++	+	+	+	-
		S.B.	+	-	+	++	-	+	-
M. lanceolata	Water	L	++	+++	+++	+++	+++	+++	+++
		R	+++	+++	+++	+++	+++	+++	+++
		S.B	++	++	+++	+++	+++	+++	+++
	DCM	L	++	+++	+++	+++	+++	+++	+++
		R	++	++	++	++	+++	+++	++
		S.B.	++	++	+++	+++	+++	+++	+++
	Methanol	L	+	++	++	+++	+++	++	++
		R	-	+	+	++	++	++	+
		S.B.	-	++	++	++	+	++	++

Key: L: Leaves, R; Roots, S.B; Stem bark, DCM; Dichloromethane.
+++ Abundant, ++ Moderate, + Trace, - Absent

4.7 *In vitro* cytotoxicity of selected plant extracts

Cell cytotoxicity against Vero E6 cell line was considered for extracts that displayed low MIC values of ≤125 mg/ml as shown in Table 4.2 and Table 4.3. As the concentration of the selected plant extracts decrease following serial dilution on the 96-well microtiter plates, the viability of the Vero cells increase as shown in Figure 4.12. This is depicted by the rate of MTT reduction quantified by measuring the absorbance of the coloured solution using spectrophotometer

Eight extracts were found to be the most active and were subjected to *in vitro* cell cytotoxicity test. Of the nine extracts: *L.cornuta* methanol leaves, *B pilosa* methanol roots and stem and *M. lanceolata* water and DCM stem bark extracts were found to be moderately toxic under fifty percent inhibitory concentration (IC_{50}) of <570 mg/ml as seen in Table 4.9. Dichloromethane extracts of *L. cornuta* roots and *M. lanceolata* leaves were less toxic to Vero E6 cell lines with IC_{50} values of 801.43 mg/ml and 684.76 mg/ml respectively. *B. pilosa* Dichloromethane extract was particularly non-toxic with IC_{50} value of 1003.54 mg/ml (Table 4.8).

Table 4.5: Effect of various plant extract on proliferation rate of Vero cell line

Concentration (µg/ml)	Proliferation Rates								
	Y1	Y2	X1	X2	Z1	Z3	X3	Z2	Y3
0.00	1.00±0.02^{a}	1.00±0.02^{a}	1.00±0.02^{a}	1.00±0.00^{a}	1.00±0.02^{b}	1.00±0.02^{a}	1.00±0.00^{a}	1.00±0.01^{a}	1.00±0.04^{a}
1.23	0.96±0.04^{a}	0.93±0.00^{a}	0.78±0.01^{a}	0.92±0.00^{a}	0.94±0.00^{a}	0.97±0.01^{a}	0.95±0.00^{ab}	0.93±0.01^{ab}	0.99±0.05^{a}
3.70	0.92±0.03^{a}	0.89±0.01^{a}	0.74±0.01^{a}	0.84±0.00^{ab}	0.93±0.01^{a}	0.95±0.01^{a}	0.92±0.00^{abc}	0.87±0.00^{ab}	0.96±0.05^{a}
11.11	0.88±0.02^{a}	0.93±0.00^{a}	0.72±0.00^{a}	0.78±0.03^{abc}	0.87±0.00^{a}	0.91±0.00^{a}	0.80±0.02^{abcd}	0.66±0.01^{bc}	0.97±0.02^{a}
33.33	0.84±0.02^{a}	0.86±0.00^{a}	0.70±0.00^{a}	0.65±0.00^{bcd}	0.85±0.00^{a}	0.89±0.00^{a}	0.62±0.00^{abcd}	0.24±0.0^{cd}	0.84±0.04^{a}
111.11	0.79±0.01^{a}	0.73±0.00^{a}	0.66±0.00^{a}	0.65±0.03^{cd}	0.83±0.00^{a}	0.81±0.00^{a}	0.49±0.03^{bcd}	0.21±0.02^{d}	0.80±0.06^{a}
333.33	0.69±0.01^{a}	0.60±0.03^{a}	0.61±0.02^{a}	0.49±0.01^{d}	0.69±0.02^{a}	0.49±0.02^{a}	0.31±0.01^{cd}	0.16±0.04^{d}	0.69±0.03^{a}
1000.0	0.05±0.01^{b}	0.54±0.05^{b}	0.46±0.01^{b}	0.24±0.00^{d}	0.32±0.02^{a}	0.12±0.01^{b}	0.08±0.00^{d}	0.05±0.01^{d}	0.29±0.01^{b}

All values are expressed as Mean±SEM. Means that do not share a letter are significantly different (*P>0.05*)

X1- *B. pilosa leaves,* X2- *B. pilosa* roots, X3- *B. pilosa* stem

Y1- *L. cornuta* leaves, Y2- *L. cornuta* roots, Y3- *L. cornuta* stem

Z1- *M. lanceolata* leaves, Y2- *M. lanceolata* roots, Z3- *M. lanceolata*

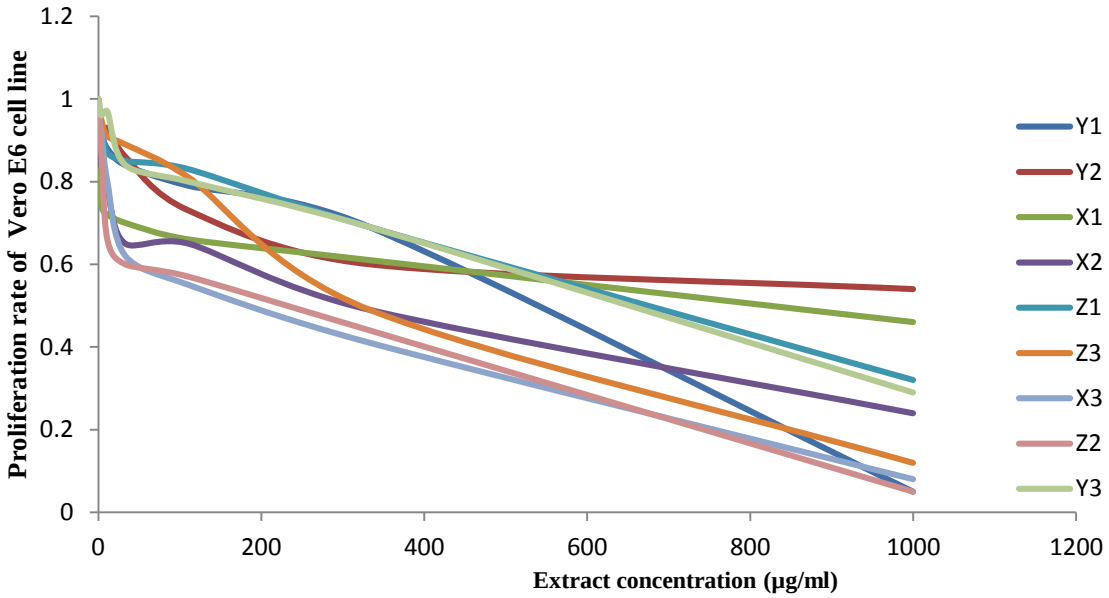


Figure 4.12: Effect of various plant extract on proliferation rate of Vero E6 cell line.

X1- *B. pilosa leaves*, X2- *B. pilosa* roots, X3- *B. pilosa* stem

Y1- *L. cornuta* leaves, Y2- *L. cornuta* roots, Y3- *L. cornuta* stem

Z1- *M. lanceolata* leaves, Y2- *M. lanceolata* roots, Z3- *M. lanceolata* stem bark

Table 4.6: *In vitro* cytotoxicity of selected plant extracts on Vero E6 cell line

Plant spp.(Plant part)	**Extract**	**IC_{50} (mg/ml)**
L. cornuta (L)	Methanol	493.5
L. cornuta (R)	Dichloromethane	800.84
B. pilosa (L)	Dichloromethane	1001.61
B. pilosa (R)	Methanol	500.74
M. lanceolata (L)	Dichloromethane	684.44
M. lanceolata (SB)	Aqueous	497.6
B. pilosa (SB)	Methanol	365.25
M. lanceolata (L)	Methanol	207.96
M. lanceolata (SB)	Dichloromethane	565.31

Key: L - leaves R - roots, SB - stem bark

4.8 Acute toxicity of selected plant extracts

Acute toxicity was investigated for extracts having MIC values as low as 125 mg/disc. The lethal dose (LD) of *M. lanceolata* (methanol and DCM) leaf extracts, *B. pilosa* (DCM and methanol) root and stem extracts, *L. cornuta* DCM (root and stem) extracts and *L. cornuta* methanol leave extracts were determined at four doses: 5 mg/kg, 50 mg/kg, 300 mg/kg and 2000 mg/kg body weight of mice respectively. Oral administration of the extracts of up to 2000 mg/kg body weight of mice, there were no deaths or signs of toxicity observed within the first thirty minutes and the subsequent 24 hours except on the mouse that had been given DCM leaf extracts of *M. lanceolata* who displayed difficult breathing, hunched posture, unkempt fur, became inactive, isolated and non-responsive to stimuli within the 24 hours of oral administration (Appendix I). The surviving mice in the test cluster gained weight in a way synonymous to those of the control surviving to day 14 when they were fed with pellets (Mice pellets UNGA® feeds) and water. The concentration of the extracts had no significant effect on the mean weight of the mice irrespective of the extracts (Table 4.10).

Table 4.7: Acute toxicity results of selected plant extracts on Swiss mice that survived

Plant species	Extract	Conc. (mg/Kg)	Survivors	% mortality
Negative control	Distilled water	10% Tween 80	3⁄3	0
B. pilosa (L)	DCM	5	3⁄3	0
		50	3⁄3	0
		300	3⁄3	0
		2000	3⁄3	0
B. pilosa (R)	Methanol	5	3⁄3	0
		50	3⁄3	0
		300	3⁄3	0
		2000	3⁄3	0
B. pilosa (SB)	Methanol	5	3⁄3	0
		50	3⁄3	0
		300	3⁄3	0
		2000	3⁄3	0
L. cornuta (L)	Methanol	5	3⁄3	0
		50	3⁄3	0
		300	3⁄3	0
		2000	3⁄3	0
L. cornuta (R)	DCM	5	3⁄3	0
		50	3⁄3	0
		300	3⁄3	0
		2000	3⁄3	0
L. cornuta (SB)	DCM	5	3⁄3	0
		50	3⁄3	0
		300	3⁄3	0
		2000	3⁄3	0
M. lanceolata (L)	Methanol	5	3⁄3	0
		50	3⁄3	0
		300	3⁄3	0
		2000	3⁄3	0
M. lanceolata (L)	DCM	5	3⁄3	0
		50	3⁄3	0
		300	3⁄3	0
		2000	3⁄3	0
M. lanceolata (SB)	Water	5	3⁄3	0
		50	3⁄3	0
		300	3⁄3	0
		2000	3⁄3	0

Table 4.8: Effect of body weight of mice after oral administration of extracts

Plant/part	Extract	Conc. (mg/Kg)	Mean weight ±SD (Kg) Day 0	Day 14
	Distilled water	10% Tween 80	20.33±2.08[a,b]	28.00±1.00[b]
B. pilosa (L)	DCM	5	18.33±1.53[a]	26.33±1.53[a,b]
		50	19.67±1.15[a,b]	28.33±3.24[a,b]
		300	20.00±2.00[b]	27.67±3.21[a,b]
		2000	20.00±3.46[a,b]	30.67±4.16[b,c]
B. pilosa (R)	Methanol	5	20.00±2.00[b]	30.33±1.53[b]
		50	21.00±1.00[b]	28.00±1.00[b]
		300	19.67±2.52[a,b]	26.67±1.53[a,b]
		2000	19.00±1.00[a,b]	24.33±0.58[a]
B. pilosa (SB)	Methanol	5	18.37±1.52[a]	28.00±1.00[b]
		50	19.67±1.52[a,b]	28.00±2.00[b]
		300	18.67±2.52[a,b]	26.33±1.53[a]
		2000	20.00±1.00[a,b]	26.33±1.53[a]
L. cornuta (L)	Methanol	5	18.67±1.52[a,b]	27.33±0.53[b]
		50	19.37±1.52[a,b]	28.33±1.53[b]
		300	21.00±1.00[b]	27.00±1.00[a,b]
		2000	20.00±1.00[a,b]	26.00±1.00[a]
L. cornuta (R)	DCM	5	18.67±1.12[a]	27.00±1.00[a,b]
		50	20.00±1.00[b]	26.00±1.00[a]
		300	20.67±1.12[a,b]	26.00±1.00[a]
		2000	19.67±2.02[a,b]	23.66±0.53[a]
L. cornuta (SB)	DCM	5	19.00±2.00[a,b]	29.00±1.00[b]
		50	21.00±1.00[b]	26.33±1.53[a,b]
		300	19.37±3.02[a,b]	27.67±1.53[a,b]
		2000	18.67±2.02[a,b]	25.00±1.00[a]
M. lanceolata (L)	Methanol	5	19.00±2.00[a,b]	29.00±1.00[b]
		50	21.00±1.00[b]	26.33±1.53[a,b]
		300	19.37±3.02[a,b]	27.67±1.53[a,b]
		2000	18.67±2.02[a,b]	23±2.65[a]
M. lanceolata (L)	DCM	5	18.67±1.52[a,b]	28.00±1.00[b]
		50	19.00±1.00[a,b]	26.67±1.53[a,b]
		300	19.67±3.22[a,b]	24.33±1.53[a]
		2000	18.37±1.52[a]	19.67±6.66[a]
M. lanceolata (SB)	Water	5	19.67±1.52[a,b]	29.00±1.00[b]
		50	20.00±2.00[a,b]	28.00±1.00[b]
		300	19.00±2.00[a,b]	25.00±1.00[a]
		2000	20.00±2.00[a,b]	24.67±1.15[a]

Means followed by the same letter within a column are not significantly different at p<0.05.

CHAPTER FIVE

DISCUSSION

The present study sought to determine the antimicrobial activity, phytochemical screening and toxicity of three medicinal plants natively used in Marakwet west sub-county, Kenya. Owing to the multidrug resistance and side effects of conventional drugs scientist's attention has been drawn majorly to investigate biologically active compounds from medicinal plants used in traditional medicine (Essawi and Srour, 2000).

5.1 Extraction from the selected medicinal plants

The comparison of the mean percentage yields of the plant material over the various extraction solvents used showed that water extracts generated the highest yields relative to other organic solvents used (DCM and methanol). This can be attributed to the fact that water has the higher polarity index (10.2) as compared to DCM and methanol (3.1 and 5.1 respectively) implying a higher potential for water molecules to interact with various polar test solutes hence ably extract more compounds from the plant material (Wabuyele, 2013). . It was also established that stem barks had higher percentage yield when compared to the roots and leaves in all solvents used. In comparing the effectiveness of the three solvents the p-value of the plant type and solvents with regard to the mean percentage yield were 0.420 and 0.899 respectively implying that the three plant types and solvents had no statistical significant effect on the percentage extract yield at 95.0% confidence level.

Phytochemicals found in the three medicinal plants studied included: terpenoids, phenols, anthraquinones, flavonoids, tannins, flavonoids and saponins. The antimicrobial activities

of the medicinal plants under investigation authenticates their claimed therapeutic potential.

5.2 Antibacterial and antifungal activity of selected extracts

The study showed a substantial difference in antimicrobial activity between extracts obtained with different solvents. Root and stem bark extracts from DCM and methanol solvents presented a better inhibition effect on the tested bacteria than extracts obtained from the leaves. This may perhaps be attributed to a higher concentration of phytochemical constituents in the roots and stem bark. The utilization of methanol and DCM as extracting solvents was noted to be effective in extracting the active compounds than water as a solvent. This possibly could be due to alcoholic aqueous environment which facilitates ease of extraction as reported by Nostro *et al*., (2000). Methanol extracts were found to be more active than the other extracts. This can be explained by their polarity index 5.1 and amphiphilic nature enabling many compounds dissolve in it with great freedom. In addition, the relatively lower boiling point of methanol (64.7 $^{\circ}$C) permits their evaporation from the extract with less damage of the phytochemical constituents.

Maesa lanceolata methanolic extracts were most active against *S. aureus* ATCC 25923 and moderately active against *S. dysentriae* and *P. aeruginosa.* The little or no activity of water extracts against most bacterial strains under investigation in this study is in tandem with prior studies which disseminate that aqueous extracts of plants showed little or no antimicrobial tendencies (Ayiegoro *et al*., 2008; Igbinosa *et al*., 2009). Methanol, DCM and aqueous extracts of *B. pilosa and L. cornuta* showed little or no activity against most tested fungi. However, *L. cornuta* methanolic leave extracts were moderately active particularly against *C. glabrata.* This tends to agree with the findings of Nagata *et al*.,

(2011) who recounted that leaves and roots of *L. cornuta* are utilized by the people of Suba sub-county, Kenya manage opportunistic diseases as a result of HIV/AIDS. On the other hand, methanol and water extracts of *M. lanceolata* particularly leave and stem bark were mostly active against most fungi species under investigation. It was noted that all extracts assayed were absolutely inactive against *A. flavus*. The difference in activity by the filamentous fungi and yeast forms and that antifungal resistance could be attributed to cell wall composition of the fungi. Filamentous fungi possess chitin and glycan while yeasts have glucans and mannan proteins. Based on this, cell wall forms a barrier that may prevent the drugs from accessing the fungal cytosol as explained by Reichhardt *et al*., (2016).

5.3 *In vitro* cytotoxicity of selected plant extracts

The reduction of the tetrazolium salt (MTT) has been widely accepted as precise calorimetric assay for assessing cellular growth. The yellow tetrazolium salt (MTT) is reduced in metabolically active cells to form insoluble purple formazan crystals which are solubilized by the addition of a detergent (van Meerloo *et al*., 2011). The reduction process occurs only when mitochondrial reductase are active and this is directly translated to the quantity of the viable cells (Riss *et al*., 2015).

Toxicity studies are very crucial in determining the efficacy of medicinal plants. As the concentration of the extract decreases following a serial dilution, the cell viability increases depicted by the optical densities that increase with decreasing extract concentration. In general, levels of cytotoxicity features to be inversely correlated to the concentration of the extract implying that the cell viability increases with decreasing concentration of the plant extracts. Five out of eight extracts were found to be moderately toxic with fifty percent

inhibitory concentration (IC_{50}) of <570 mg/ml. Dichloromethane extracts of *L. cornuta* roots and *M. lanceolata* leaves were less toxic to Vero E6 cell lines with IC_{50} values of 801.43 mg/ml and 684.76 mg/ml respectively. *B. pilosa* Dichloromethane extract was particularly non-toxic with IC_{50} value of 1003.54 mg/ml.

In the study, the methanol extracts of *L. cornuta* leaves, *M. lanceolata* leaves, *B. pilosa* stem bark and aqueous stem bark extracts of *M. lanceolata* all having an IC_{50}<500 mg/ml were found to be moderately cytotoxic. However, these extracts did not display any acute toxicity *in vivo*. The reason for this could be attributed to the complex immune system of cells capable of metabolizing and detoxifying chemicals to less toxic compounds. This ability is generally expressed to a little extent in cultured cells that culminates in limited detoxification of chemicals (Walum, 1998). It can therefore be concluded that *in vitro* cytotoxicity cannot directly infer *in vivo* acute toxicity. The fifty percent cytotoxic concentration CC_{50} values of the three plants indicate that the extracts were not toxic to the Vero cells and thus considered safe to use as herbal remedies.

5.4 Acute toxicity of selected plant extracts

Various scientific reports disseminate that many plants used as traditional medicine and food are potentially toxic and there is little indication of scientific examination done on such plants (Schimmer *et al*., 1994). Screening of such plants is vital for determining their safety on uses and that *in vivo* toxicity studies on animals are critical in determining the potential toxic effects of phytochemicals in human beings subjected to near fatal doses. There was no mortality observed within 24 hours in Albino mice that received *L. cornuta. M. lanceolata* and *B. pilosa* extracts in all dose levels. However, mice had physical signs

such as crowding in a cage, unkempt fur and moderate activity especially with regard to the highest concentration tested (2000 mg/kg body weight). This incident was more pronounced in dichloromethane leave extract of *M. lanceolata*. On the same note, there was an increase in weight in all the groups of mice treated with the selected plant extracts under dose levels and that their weights were not significantly different from that of control mice ($p<0.05$).

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

The present study established that water extracts of the three medicinal plants gave the highest mean percentage yield of 3.63% followed by dichloromethane 2.91% and lastly methanol at 2.70%. Generally, of the three plants *M. lanceolata* had the best antibacterial and antifungal activity. DCM, methanol stem bark and root extracts showed a higher antibacterial activity than extracts derived from the leaves. Stem bark methanolic extracts of *M. lanceolata* displayed a higher antibacterial activity of 21 mm and 3.90625 mg/ml MIC value relative to the gentamicin giving a mean inhibition zone diameter of 13 mm.

Extracts of *B. pilosa* and *L. cornuta* demonstrated least antifungal activity against the fungal strains used. However, most extracts of *M. lanceolata* particularly from the roots and stem bark were very active and that aqueous root and stem bark extracts were highly active against *Candida glabrata* with zone diameter inhibitions 23.0 mm and 20.0 mm respectively as compared to that of Fluconazole 18.3 mm and 19.0 mm respectively. This indicates that *M. lanceolata* can be exploited for therapeutic use against both bacterial and fungal pathogens causing infections.

In both cell and acute toxicity, it was observed that both DCM, water and methanol extracts of *M. lanceolata, B. pilosa* and *L. cornuta* were not toxic and that at the stated concentrations they are safe for medicinal uses.

Phytoconstituents such as terpenoids, phenolics, anthraquinones, flavonoids or tannins as reported elsewhere are responsible for antimicrobial activities in plants. The antibacterial and antifungal activities of the medicinal plants under investigation validates their support of their claimed therapeutic potential because they were detected and were found active to varying degrees.

6.2 RECOMMENDATIONS

- Some extracts of *M. lanceolata, B. pilosa* and *L. cornuta* have both antifungal and antibacterial potential thus isolation and purification of the active compounds and determination of their bioactivity is paramount. Doing so culminates to identification of new range of active principles vital for the management of fungal and bacterial infections.
- Chronic toxicity should be carried out on the crude extracts to determine their safety profiles.
- The pursuit for new antimicrobials is a continuous process since the contemporary drugs have limitations. Medicinal plants are one of these sources and therefore sustainable use and conservation of these plants is highly recommended.

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APPENDICES

Appendix 1: Preparation and extraction of crude extracts

a) Ground plant material

b) Filtering of DCM and methanol extracts

c) Removal of excess DCM and methanol using Rotor evaporator

d) Organic extracts left to dry

e) Extraction of aqueous extracts on freeze dryer

f) Behavioral observation of mice

g) Behavior of mice after oral administration of the extracts at 2000mg/kg

Appendix 2: Analysis of Variance for mean percentage extract yield for different solvents

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-value*
MAIN EFFECTS					
A: Plant type	9.83887	2	4.91943	1.09	0.4203
B: Solvents	0.983267	2	0.491633	0.11	0.8998
RESIDUAL	18.1347	4	4.53367		
TOTAL (CORRECTED)	28.9568	8			

Df – degree of freedom