# ANTIMICROBIAL ACTIVITIES OF SECONDARY METABOLITES FROM *Vernonia adoensis* AND *Ocimum kilimandscharicum* MEDICINAL PLANTS AND ASSOCIATED ENDOPHYTES FROM KAKAMEGA FOREST IN KENYA

**BY**

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## DECLARATION

This thesis is my original work prepared by no other than the indicated sources and support and has not been presented elsewhere for a degree or any other award. No part of this thesis may be produced without prior written permission of the author and Maseno University.

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## DEDICATION

I dedicate this thesis to my late father Nyongesa, mum Hannah, wife Josephine, daughters Sheila, Sharon, Hannah, Rachael, Macrine and my son Benjamin for their unrelenting support and encouragement in my academic journey. God bless you all.

## ABSTRACT

Medicinal plants have been used for the treatment of different ailments in human and animal health systems for many years. Approximately over 80% of rural and urban African populations use plant-based synthetic products for primary healthcare. However, the widespread and indiscriminate use of antibiotics has led to clinical resistance of previously sensitive microorganisms. Despite plants and associated endophytes being rich sources of secondary metabolites with antimicrobial potential, little is known about the antimicrobial potential of many medicinal plants including *Ocimum kilimandscharicum* and *Vernonia adoensis* in Kakamega Forest. This study investigated the antimicrobial activities of the secondary metabolites from the two plants and their associated endophytes, by specifically screening the secondary metabolites for phytoconstituents and determining their antimicrobial activities. An experimental design using a purposive sampling method was used in the study. The crude extracts from the plants and endophytes were obtained by standard extraction methods. The antimicrobial activities against selected bacteria (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*) and fungi (*Candida albicans*) were determined using the agar well diffusion and micro dilution methods. The choice of the pathogens was on the basis of the diseases the plants treat in the traditional medicine. Kruskal-Wallis was utilized to determine the difference in the diameter of the inhibition zones of the extracts against the susceptible microorganisms. The qualitative phytochemical screening showed that the plants had alkaloids, terpenoids, tannins, steroids, saponins and phenols. Both plants also showed bacteria (*Bacillus* sp) and fungi (*Alternaria* sp and *Phomopsis* sp) as culturable endophytes. The endophytic metabolites from both plants showed the presence of terpenoids, flavonoids and alkalloids. The extracts from the plants significantly inhibited growth of susceptible bacteria (*Staphylococcus aureus*; $p = 0.0138$ and *Escherichia coli*, $p = 0.223$ and from endophytes (*Klebsiella pneumoniae*; $p = 0.0211$, *Escherichia coli*, $p = 0.0226$ and *Candida albicans*, $p = 0.001$). Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) results suggested that the metabolites can be potential antimicrobial agents against the pathogenic microorganisms. The study results showed that the plants and endophytes possess bioactive chemical compounds with antimicrobial potential. The findings are useful in understanding the antimicrobial potential of the medicinal plants in managing the emerging drug resistance in pathogenic microorganisms. From the study, there is need to conduct a detailed bioassay-guided phytochemical studies, profile the endophytes in the plants, characterize the secondary metabolites from these plants and endophytes and determine the antimicrobial potential of the purified compounds from the two medicinal plants.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| **AIDS** | Acquired Immunodeficiency Syndrome |
| **ATCC** | American Type Culture Collection |
| **BaCB** | Bacterial Colony |
| **BC** | Before Christ |
| **BCF** | Brown Fungal Colony |
| **DMSO** | Dimethyl sulfoxide |
| **GC** | Gas chromatography |
| **GLC** | Gas Liquid Chromatography |
| **GC/MS** | Gas Chromatography/Mass Spectrometry |
| **$H_2S$:** | Hydrogen Sulfide |
| **$H_2SO_4$:** | Sulfuric Acid |
| **LSC** | Liquid Scintillation Counter |
| **MBC:** | Minimum Bactericidal Concentration |
| MHA | Mueller Hinton Agar |
| **MDR:** | Multi-drug Resistant |
| **MFC**: | Minimum Fungicidal Concentration |
| **MIC:** | Minimum Inhibitory Concentration |
| **NA:** | Nutrient Agar |

**PDA:** Potato Dextrose Agar

**SEM:** Standard Error of the mean

**WCF:** White Fungal Colony

**WHO:** World Health Organization

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# CHAPTER ONE

# INTRODUCTION

## 1.1 Background Information

The use of medicinal plants as a source of relief from illness is as old as mankind. Available records on clay tablets in cuneiform in Mesopotamia dating back to 2600 B.C indicate that some of the plant extracts such as oils from Cedrus species, Cypress, Licorice and Myrrh were used and are still in use today in traditional medicine in the management of microbial infections (Newman *et al*., 2007), although with unconfirmed efficacy due to lack of rigorous controlled clinical trials (Martin, 2003). The conventional drugs that have always provided effective antibiotic therapy for majority of the infections are increasingly meeting microbial resistance due to indiscriminate use (Strobel *et al*., 2003).This situation is quickly paving way to the emergence of multidrug resistant (MDR) pathogens (Bandow *et al*. 2003) and the appearance of strains with reduced susceptibility as well as, undesirable side effects of certain antibiotics. This resistance problem demands that a renewed effort be made to screen various medicinal plants for their potential antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant.

Traditionally used medicinal plants produce a variety of compounds of known therapeutic properties (Nautiyal *et al.*, 2008; Harborne, 1998) that can either inhibit the growth of pathogens such as bacteria (bacteriostatic) or kill them (bactericidal) with no or least toxicity to host cells.

It is on this basis that in recent years, antimicrobial properties of medicinal plants are increasingly being reported from different parts of the world (Vadlapudi, 2010).

Secondary metabolites from herbal extract mixtures such as tannins, terpenoids, alkaloids and flavonoids have proven to possess *in vitro* antimicrobial properties (Cowan, 1999) and may therefore serve as models for discovering bioactive products with diverse chemical structures and mode of action. These chemicals could therefore be active against microbial pathogens.

Although plants have been an important source of natural bioactive compounds, endophytes which are microbes that colonize living, internal tissues of plants without causing any immediate negative effects (Bacon *et al*, 2000) have also provided a broad variety of bioactive secondary metabolites with unique structures. These structures include alkaloids, benzopyranones, flavonoids, phenolic acids, quinines, steroids, terpenoids, tetralones, xanthones, and others (Tan *et al,* 2001a). These bioactive metabolites are currently used as agrochemicals, antibiotics, immunosuppressants, antiparasitics, antioxidants, and anticancer agents ( Strobel, 2003). Some of these bioactive compounds have an antifungal activity against *Candida albicans* (Yu *et al.* 2010).

Incidentally, endophytes have remained a poorly investigated group of microorganisms with a rich source of bioactive and chemically novel compounds with potential for exploitation in a wide variety of medical, agricultural, and industrial fields. This study therefore focused on two medicinal plants that have for a long time been used by the natives of Kakamega to manage some illness among the population and have been widely recommended by the traditional healers in the traditional medicine around the Kakamega Forest. The choice of the plants for the study was based on the responses by the people and traditional healers and medicine men that use

them. The plants were *Vernonia adoensis* and *Ocimum kilimandscharicum.* The *Vernonia* genus has many species and members of the genus are widely used as food and medicine (Toyang *et al.*, 2013). Majority of the *Vernonia* species have been reported to possess medicinal properties in the management of human and animal diseases. These plants possess sesquiterpene lactone with bioactivity in antiplasmodial, antileishmanial, antischistosomial, cytotoxicity, antimicrobial and anti-inflammatory assays. *Vernonia amygdalina* is the most promising species as a nutraceutical against diabetes and malaria (Toyang *et al*., 2013). Ngule, 2013 has shown that methanol extracts of *Vernonia adoensis* leaves from the Nandi forest have antimicrobial activities against clinical cases of bacteria. Little is known about the phytochemical profile and the antimicrobial activity of the *Vernonia adoensis* extracts from the diverse ecosystem of the Kakamega forest in Kenya which this study addressed. Whereas methanol-water was used in the extraction of the metabolites from the leaves of the *Vernonia adoensis* from the Nandi forest; more solvents (hexane, chloroform and ethanol) were used to extract the metabolites of the *Vernonia* and *Ocimum* spp from the Kakamega forest in the leaves and stems.

The genus *Ocimum*, member of Lamiaceae family comprises of almost 200 species of herbs and shrubs (Charles *et al.*, 1990) and is graded high among some of the astonishing herbs for having tremendous medicinal potency. This genus is widespread over Asia, Africa, Central and Southern America. The genus *Ocimum* is cultivated for its extraordinary essential oil which displays many therapeutic usages such as in medicinal application, herbs, culinary perfume for herbal toiletries, aroma therapy treatment and as flavoring agent (Groom, 1997; Kashyap *et al.* 2011). *Ocimum kilimandscharicum* is an important aromatic medicinal plant in Kenya(Paton *et al.*, 1996). It is a perennial evergreen shrub having oblong, ovate green colored leaves (0.5-5 m), oppositely arranged having pubescent leaf surface, narrow at the base and deeply serrated. One

seeded fruits are indehiscent type found in clusters, hermaphrodite flowers are found in clusters, tap roots are deep and soft wooded. The leaves contain aromatic oils, which represents the essence of the plant. The essential oil is extracted using distillation, expression or solvent extraction methods. The oil constitutes liquid oil and white solid crystals, where the pure crystals possess a characteristic odor and taste of natural camphor ((Saha *et al*., 2011).

Extracts of different solvents from *Ocimum gratissimum*(Adebolu *et al*., 2005)) showed antibacterial activity against clinical cases of *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi* and *Salmonella typhimurium*, pathogenic bacteria that cause diarrhea. In another study, the volatile oils from *Ocimum basilicum* exhibited considerable inhibitory effect against all the tested organisms(Baratta *et al*. 1998). Currently there is no known information about the phytochemical profile and the antimicrobial potential of the extracts of the *Ocimum kilimandscharicum* of the Kakamega forest considering that plant secretions and secondary metabolites vary not only among taxa but among kinds of communities and geographic regions as well (Lewinsohn, 1991). In realizing the objectives of the study,, secondary metabolites from the two plants and the endophytes isolated from them were screened for their phytoconstituents composition. These bioactive compounds were then tested against susceptible bacteria and fungi that included *Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa and Candida albicans* to determine their antimicrobial potential. The choice of the tested microorganisms was on the basis of the diseases the plants are used to treat.

## 1.2 Statement of the Problem

The use of the herbal concoctions/decoctions in traditional medicine to manage microbial diseases is well studied (Kakudidi *et al*. 2015)**.** There is data on the wider applications of natural products of plant origin. Further, although studies show that medicinal plants at varied times produce metabolites that possess antimicrobial potential; there is need to examine the metabolites from the medicinal plants of the Kakamega forest. Substantial study data indicate that endophytes produce many secondary metabolites with antimicrobial potential that may have some similarities with those produced by the host plant (Strobel *et al.,* 2003). The distribution and concentration of the bioactives in the endophytes is dependent on the geographical location, tissue type and age of the host plant. Though currently there is a lot of research effort to screen the antimicrobial potential of these secondary metabolites from the endophytes of most medicinal plants in many other regions, there is need to bio prospect the same in the medicinal plants of the Kakamega Forest. With increased reports on microbial resistance on most of the commonly used antibiotics (WHO, 2009), the search for an effective therapy from the medicinal plants of the natural sources necessary. So far there is no study that has addressed the antimicrobial activities of the secondary metabolites from the *Ocimum kilimandsharicum* and *Vernonia adoensis* of the rich and diverse ecosystems. This study therefore investigated the antimicrobial activities of the secondary metabolites from the two plants and their associated endophytes of the Kakamega Forest.

## 1.3 Objectives of the study

### 1.3.1 General Objective

To evaluate the antimicrobial activities of solvent extracts from *Vernonia adoensis* and *Ocimum kilimandscharicum* medicinal plants and their associated endophytes of Kakamega Forest in Kenya.

### 1.3.2 Specific Objectives

1. To determine the phytochemical substances present solvent extracts from the *Vernonia adoensis* and *Ocimum kilimandscharicum* medicinal plants of the Kakamega forest in Kenya.
2. To determine bioactive secondary metabolites present in the solvent extracts from the endophytes of the *Vernonia adoensis* and *Ocimum kilimandscharicum* medicinal plants of the Kakamega forest in Kenya
3. To determine the antibacterial and antifungal activities of the solvent extracts from the plants and the associated endophytes of the *Vernonia adoensis* and *Ocimum kilimandscharicum* medicinal plants of the Kakamega forest in Kenya.

## 1.4 Hypotheses

1. The solvent extracts from the *Vernonia adoensis* and *Ocimum kilimandscharicum* medicinal plants contained phytochemical substances.
2. The endophytes isolated from the *Vernonia adoensis* and *Ocimum kilimandscharicum* medicinal plants produce bioactive secondary metabolites.
3. There is no difference in antibacterial and antifungal activity of the extracts from *Vernonia adoensis* and *Ocimum kilimandscharicum* and the associated endophytes.

## 1.5 Significance of the study

The results significantly reveal that the plants and their endophytes have phytochemicals that exhibit antimicrobial activity. These novel products are essential compounds that could be used as effective drugs that may be useful in the management of the ever microbial infections. The isolation of the endophytic extracts gives a clue of the potential of the endophytes from the two medicinal plants to produce antimicrobial substances that can be used to treat pathogenic microorganisms. The discovery of the potential antimicrobial substances from both plants and endophytes calls for the concerted efforts in conservation of the two plants which are fast becoming extinct from the forest.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Use of Medicinal Plants in Disease Management

Infectious diseases are considered a major threat to human health because of the unavailability of vaccines to prevent most of them or limited chemotherapy to effectively manage them. They account for approximately one half of all deaths in tropical countries (Iwu 2014). The infections which ranked 5$^{th}$ in 1981, became the 3$^{rd}$ leading cause of death in 1992, with an increase of 58% (Pinner *et al*. 1996). Most of the current antibiotics that have been in use have considerable limitations in terms of antimicrobial spectrum and side effects. The widespread overuse of these antibiotics has led to increased clinical resistance of previously sensitive microorganisms and to the occurrence of uncommon infections (Grayer *et al*. 2002). With the advent of these ever-increasing resistant bacteria and fungi strains, there is a corresponding rise in the universal demand for natural antimicrobial therapeutics (Assob *et al.* 2011). Herbal medicines that have widely been used in traditional medicine are now forming an integral part of the primary health care in many countries. These plants therefore constitute a reservoir of new antimicrobial substances yet to be discovered.

In Africa, the use of herbal medicines represents a long history of human interactions with the environment. The plants that have been used contain a wide range of substances that can be used to treat chronic as well as acute infectious diseases (Diallo *et al*., 1999). These plant extracts and /or phytochemicals that have been tested for activity against drug-resistant strains or can act as antibiotic resistance inhibitors are divided into three categories of plant materials with general antimicrobial activity against different microorganisms including some drug-resistant strains,

plant materials with specific antimicrobial activity against drug-resistant strains and plant materials which restore the effectiveness of antimicrobial agents and/or inhibit drug resistance mechanisms (Oyedemi *et al.* 2016). A study by Madikizela *et al*., (2013) on the *in vitro* antimicrobial activity of extracts from plants used traditionally in South Africa to treat tuberculosis and related symptoms showed good antimicrobial activity against at least one or more of the bacterial strains tested. The results from this study indicated that the good antimicrobial properties could form a basis for further pharmacological and phytochemical investigation and validate the traditional use of the plants in the treatment of respiratory diseases such as tuberculosis.

In Kenya, a study to investigate the antimicrobial activity and presence of active phytochemical compounds in *Vernonia glabra*; a plant used by herbalists in parts of Kenya for the treatment of gastrointestinal problems showed that it contains phytochemicals of medicinal properties and justify the use of the plant in traditional herbal medicine for the treatment of microbial based diseases (Kitonde, 2013). In another study carried out on seven medicinal plant extracts traditionally used in Kenya, mainly for management of infectious conditions showed significant antibacterial activity *(Wagate et al.* 2010). A methanol-water extract from *Vernonia adoensis* plant from the Nandi Forest to evaluate antibacterial activity from the leaves against *Salmonella typhi, Klebsiella* sp, *Bacillus cereus, Streptococcus pyogenes, E. coli, Proteus vulgaris and Enterobacteria erogenes* showed that the extract can be used to control *B. cereus, Klebsiella sp., Streptococcus pyogenes* and *Proteus vulgaris* (Ngule *et al.*, 2013). A related study using different solvents to obtain extracts from the medicinal plants from the Kakamega forest may provide alternative products therapeutic products for the management of microbial infections especially

from diverse and less studied environments. The current research especially targeted the unique habitats from diverse ecological ecosystems for candidate natural antimicrobials.

### 2.2 Plant Extracts and Phytochemical Screening

Many higher plants accumulate extractable organic substances in quantities sufficient to be economically useful as chemical feedstocks or raw materials for various scientific, technological, and commercial applications (Kheyrodin 2009*).* For the sake of convenience, plant chemicals are often classified as either primary or secondary metabolites (Balandrin *et al.* 1985); proteins and nucleic acids are generally excluded from this classification. Primary metabolites are substances widely distributed in nature, occurring in one form or another in virtually all organisms. In higher plants such compounds are often concentrated in seeds and vegetative storage organs and are needed for physiological development because of their role in basic cell metabolism *(*Cseke *et al.* 2006;Berenbaum, 1995). As a general rule, primary metabolites obtained from higher plants for commercial use are high volume-low value bulk chemicals. They are mainly used as industrial raw materials, foods, or food additives and include products such as vegetable oils, fatty acids (used for making soaps and detergents), and carbohydrates (for example, sucrose, starch, pectin, and cellulose).

The compounds have no apparent function in a plant's primary metabolism but often have an ecological role; they are pollinator attractants, represent chemical adaptations to environmental stresses, or serve as chemical defenses against microorganisms, insects and higher predators, and even other plants - allelochemics (Mann *et al.* 2012).Unlike primary metabolite, the secondary metabolites are frequently accumulated by plants in smaller quantities than are primary metabolites. These secondary metabolites include tannins, terpenoids, alkaloids and flavonoids. They tend to be synthesized in specialized cell types and at distinct developmental stages,

making their extraction and purification difficult (Waksmundzka,*et al.,* 2008). As a result, secondary metabolites that are used commercially as biologically active compounds (pharmaceuticals, flavors, fragrances, and pesticides) are generally higher value-lower volume products than the primary metabolites and are considered as specialty materials or fine chemicals (Balandrin *et al*. 1985).

Research in phytochemical investigation has revealed the presence of tannins, saponins, alkaloids, glycosides, flavonoids and essential oils from plant extracts (Dash *et al.,* 2008). Inhibition tests done have often varied from phytochemical nature which then needs to concentrate each of the phytochemicals, quantified and the structure determination. Studies regarding the variation in the biological activities of extracts obtained from different extraction techniques emphasize the importance of selecting the suitable method (Annegowda *et al*. 2012)**.** Currently, conventional extraction techniques such as maceration and Soxhlet extraction were used along with several novel extraction techniques such as ultrasonic, accelerated solvent extraction and microwave assisted extraction to accelerate the extraction of bioactive compounds from plants (Wang *et al*. 2012). This study employed the Soxhlet extraction method that utilized many solvents with an increasing polarity thus enhancing harvest of the metabolites from the plants and was also a preferred extraction method where boiling of the herbs is done in the traditional medicine besides maceration another very popular method.

In this study two medicinal plants were selected on the basis of their use in traditional medicine to cure infectious diseases including urogenital tract infections and they included; *Vernonia adoensis (*known in local language as Msululitsa) and *Ocimum kilimandscharicum, (*known

locally as Mwonyi) for their phytochemical screening and antimicrobial activity against microbial pathogens (Akerele 1991; Kokwaro 2009).

### 2.2.1 *Vernonia adoensis*

The *Vernonia* genus has about one thousand (1000) species and members widely distributed in most tropical and subtropical countries where they are often used by the locals as food and medicine. They have long been used in traditional medicine to treat various types of diseases (Ntie-Kang *et al.* 2014). A total of 109 *Vernonia* species have been reported to have medicinal properties. One hundred and five (105) plants have been linked to the treatment or management of 44 human diseases or health conditions. Plants of the genus also feature in ethnoveterinary and zoopharmacognostic practices (Jeruto *et al*. 2008). A total of 12 *Vernonia* species have been identified to be used in ethnoveterinary medicine while 2 species are used in self-medication practices by chimpanzees and gorillas (Toyang *et al.,* 2013). In recent years, the interest in the plant-based medicine has increased worldwide.

*Vernonia adoensis* in the family Asteraceae has been known to have healing potential with many therapeutic uses in the practice of traditional medicine (Cousins *et al*., 2002).Every part of the plant can be used medicinally (Iwu *et al.,* 2014; Giday *et al.* 2003). Among the diseases the plants have been used to treat are disorders including inflammation, malaria, fever, worms, pain, diuresis, cancer, and various gastro-intestinal disorders and in the management of wounds (Sasidharan *et al.* 2010). The roots of *Vernonia adoensis* have been used traditionally mainly for the treatment of sexually transmitted diseases such as gonorrhea by the residents of Rift Valley and Western part of Kenya (Kokwaro 1991). The plant leaves have also been used in the treatment of malaria (Stangeland *et al*. 2010) The decoction of the roots mixed with the back of

other trees is used in the treatment of heart and kidney problems (Ngule *et al*., 2013). The plant leaves and stem ash are also commonly used in wounds management among the Bukusu tribe in Bungoma County of Western Kenya (Courtesy of Indigenous knowledge among the Bukusu. A study is therefore required to screen the secondary metabolites produced by the plant and the endophytes hosted by the plant and to determine the antimicrobial activities of these bioactive metabolites.

### 2.2.2 *Ocimum kilimandscharicum*

The genus *Ocimum*, member of Lamiaceae family comprises of almost 200 species of herbs and shrubs (Charles and Simon 1990)and is graded high among some of the astonishing herbs for having tremendous medicinal potency. There are large numbers of distinct species and varieties which fall in this genus (Groom 1997)**.** Genus *Ocimum* is widespread over Asia, Africa, Central and Southern America. The genus *Ocimum* is cultivated for its extraordinary essential oil which displays many therapeutic usages such as in medicinal application, herbs, culinary perfume for herbal toiletries, aroma therapy treatment and as flavoring agent (Groom 1997). Due to the difficulties in identifying the species, it has been concluded that identification can be optimized by combined analysis of morphological traits, essential oil composition and molecular markers as well as biological activity (Massimo *et al.*, 2004). *Ocimum kilimandscharicum* is an important aromatic medicinal plant in Kenya (Paton *et al.*, 1996). It is a perennial evergreen shrub having oblong, ovate green colored leaves (0.5-5 m), oppositely arranged having pubescent leaf surface, narrow at the base and deeply serrated. One seeded fruits are indehiscent type found in clusters, hermaphrodite flowers are found in clusters, tap roots are deep and soft wooded. The leaves accommodate aromatic oils, which represents the essence of the plant. Traditionally, extracts of *Ocimum kilimandscharicum* have been used to mitigate many disorders in East Africa

comprising remedy of coughs, colds, measles, abdominal pains, diarrhea, insect repellent, particularly against mosquitoes and storage pest control (Kokwaro 1991), Jembere *et al.* 1995). The essential oils obtained from this plant as repellent against biting insects and malaria vector have been practiced in North-Eastern Tanzania for centuries (Kweka *et al*. 2008). The essential oil is extracted using distillation, expression or solvent extraction methods. The oil constitutes liquid oil and white solid crystals, where the pure crystals possess a characteristic odor and taste of natural Camphor (Saha *et al.,* 2011). The hydro-distilled essential oil of *Ocimum kilimandscharicum* studied by capillary gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) has been reported to accommodate oxygenated monoterpenes (95.8%), represented by camphor (64.9%), limonene (8.7%), camphene (3) (6.4%) and (E)- -ocimene (3.0%) (Klima´nkova *et al,* 2008,Simon *et al.,* 1990,Padalia *et al*., 2011). The essential oil of *Ocimum kilimandscharicum* investigated by LSC, GLC and GC-MS and was found to contain 62% 1, 8-cineole 16 oxygen containing compounds and 14 monoterpenes hydrocarbons including limonene (Grayer, 2002)**.** The essential oil of *Ocimum kilimandscharicum* grown in Indiana region of USA has been analyzed by GC and GC/MS and 17 constituents have been identified in oil. The essential oil content varied between the leaves (0.77-1.12% dry wt. basis) and the flowers (1.96-2.8% dry wt.). Oil composition was similar between the leaves and flowers with linalool as the major constituent, leaves (41.94%), flowers (58.85%) (Hussain *et al.* 2012).

In Kenya, using modern science and technology, a new brand of medicines called Naturub® was developed from purified extracts of *Ocimum kilimandscharicum* based on the traditional knowledge and practices (Soni *et al.* 2012). Naturub® is registered as a medicine and is certified and registered as the first natural product by the Pharmacy and Poisons Board of Kenya. It is sold widely in corporate retail chains in Kenya. Its balm is

used for alleviating flu, cold, chest congestion, aches and pain, insect bites and muscular pain.

### 2.3 Antimicrobial Natural Products from Endophytes

Natural products are metabolites from micro-organisms, plants or animals (Baker *et al.* 2013) and have been the traditional sources of drugs. In many cases, these natural products have served as sources of lead molecules yielding many synthetic drugs. The world's first billion-dollar anticancer-drug, paclitaxel (Taxol) is one outstanding example of a natural product from Yew tree, *Taxus wallachiana* (Schulz, 2004). Strobel (2003) reported that endophytic fungus (*Pestalotiopsismicrospora*) found in Yew tree is also able to produce Taxol. The classical immunosuppressive cyclosporine isolated from the endophytic fungus (*Tolypocladiuminflatum*) further increased the importance and significance of endophytes.(Strobel *et al*. 1993; Qadri *et al*. 2013). Endophytes are viewed as a great source of bioactive natural products. For example several endophytic bacteria (EB) have been shown to produce natural products like phytohormones, low molecular weight compounds, enzymes, siderophores, and antibiotics (Subhash J Bhore, Ravichantar, and Loh 2010). The majority of them (EB) do produce different kinds of antibiotics; and in fact, they are one of the untapped potential sources of novel antibiotics. Ecomycins, Pseudomycins, Munumbicins, Kakadumycins are some examples of the novel antibiotics produced by endophytic bacteria (Christina *et al*., 2013).

Studies show that endophytic fungi, isolated from the rhizomes of a traditional medicinal herb; *Paris polyphylla* var. *yunnanensis*, demonstrated strong antibacterial activities against four Gram-negative bacteria (*Escherichia coli, Xanthomonas vesicatoria, Agrobacterium tumefaciens* and *Pseudomonas lachrymans*) and two Gram-positive bacteria (*Bacillus subtilis, Staphylococcus haemolyticus*)(Xu *et al*. 2008). About 353 endophytic fungi, with no known compound isolated, were separated from medical plants (*Cyrtomium, Ophiopogon*). The

proportion of the compound inhibiting the growth of *Alternaria solani* and *Botrytis cinerea* was 15.9% and 11.3%, respectively (Jiang *et al.,* 2002). Phongpaichit *et al.* (2006) obtained the fermentation broths of endophytic fungi from plants of *Garcinia* and tested the antimicrobial activity through the agar diffusion method against *Staphylococcus aureus, Candida albicans* and *Cryptococcus neoformans.* The results showed that 70 strains of the total 377 (18.6%) displayed antimicrobial activity against at least one pathogenic microorganism. A mass of endophyte studies are still at the screening stage, and the active ingredients need to be isolated (Subhash *et al.*, 2010). Information of endophytes in *Ocimum* and other species of *Vernonia* is scanty and more research is therefore required to discover endophytic metabolites as potential ingredients for novel drugs.

# CHAPTER THREE

# MATERIAL AND METHODS

## 3.1 Study Site

The selected plants were collected about 100 metres radius from the perivery of the Kakamega forest. The Kakamega Forest stretches from 0°10"-0°21"N and longitude 34°47'-'34°58" E. It is located in Kakamega and Vihiga Counties in the former Western Province of Kenya and lies within the Lake Basin North of the Equator. The reserve comprises of 5 blocks namely Malava (719 ha), Bunyala (825 ha), Kaimosi (200 ha), Maragoli (720 ha) and the main forest block Kakamega comprising of Isecheno (14.500 ha), Kisere (471 ha) and Buyangu (3,997 ha). An experimental study design with a purposive sampling technique was employed in the study.

## 3.2 Identification of Potential Plants for Antimicrobial Analysis

An inventory of plant species with reported activity against pathogenic microorganisms was established through a multi-step approach to select the candidate plants (Nath *et al.*, 2015). This included consultations with renowned ethnomedical/ethnoveterinary practitioners and other traditional healers in the Western Province who had been interviewed prior to the survey based on their active roles in ethnomedicine/ethnoveterinary. A questionnaire as a tool was used to collect information from the relevant institutions (Appendix). Diseases that the plants treat among the local people were established. Literature search and interrogation of available online databases such as PRELUDE and NAPRALART for available published information on general antimicrobial potential of local plants was performed. There were also field surveys that included established ethnobotanical research units at various institutes including Kenya Forest Service, Kenya Forest Research Institute and Kenya National Museums for the records of the plants, part

used and preparation and dosing procedures and levels. Where possible, pictures of the plants were taken as aids to the botanical identification of the plants. Samples of the plants were taken and submitted to the Biological Sciences, Masinde Muliro University of Science and Technology, Botany Laboratory for identification by a plant taxonomist.

### 3.3 Collection and Extraction Methods of Phytochemicals

Based on ethnobotanical uses, the candidate medicinal plants were selected and appropriate samples (leaves and stems) collected (sufficient to provide 100g ground powder) separately in self-sealing (zip) plastic sample bags and promptly transported to the laboratory on ice in an ice box. The samples were then stored at $4^{o}$C until isolation as recommended by (Strobel *et al.,* 2003). Only healthy and disease free plant parts were selected in order to minimize the presence of plant pathogenic species, and to prevent the isolation of localized pathogenic endophytic microorganisms. The plant samples were divided into two parts. The first parts were processed within 12 hours for endophyte isolation and the second part was used for crude extracts upon drying at room temperature in the dark to avoid decomposition of the phytochemicals. Dried and finely ground plant materials were extracted by Soxhlet extraction technique using different solvents successively with increasing gradient polarity (hexane, chloroform, and ethanol). This was done by transferring 100g of the finely ground plant material to a porous cellulose thimble and placing it in an extraction chamber suspended above a flask containing the respective solvent and below a condenser. Each successive extraction step took six hours. The color and percentage yield were noted. The extracts were then completely evaporated by vacuum distillation (Rotor Vapour- Sigma Aldrich) and stored for analysis (Sahaya *et al.,* 2012)**.** These techniques of extraction were preferred to traditional methods due to their efficiency and high yield of the targeted metabolites.

### 3.4 Phytochemical Analysis

#### 3.4.1 Phytochemical Screening

Phytochemical screening was performed to assess the qualitative chemical composition of different samples of crude extracts using commonly employed precipitation and coloration reactions to identify the major secondary metabolites like alkaloids, flavonoids, glycosides, Proteins, phenolic compounds, saponins, starch, steroids, tannins and terpenoids as described by Harborne (1998).

##### 3.4.1.1 Test for tannins

About 0.5g of the dried powdered samples was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black coloration.

##### 3.4.1.2 Test for phlobatanins

Deposition of a red precipitate when an aqueous extract of each plant was boiled with 1% aqueous hydrochloric acid was taken as evidence for the presence of phlobatanins.

##### 3.4.1.3 Test for saponins

About 0.5 g of the plant extract was shaken with water in a test tube. Frothing, which persist on warming was taken as a preliminary evidence for the presence of saponin. Few drops of olive oil was added to 0.5 g of the extract and vigorously shaken. Formation of soluble emulsion in the extract indicates the presence of saponin.

##### 3.4.1.4 Test for flavonoids

Three methods were used to determine the presence of flavonoids in the plant sample (Sofowora 1993; Harborne 1998) 5 ml of dilute ammonia solution was added to a portion of the aqueous

filtrate of each plant extract followed by addition of concentrated sulphuric acid ($H_2SO_4$). A yellow coloration that disappeared on standing observed in each extract indicated the presence of flavonoids. Few drops of 1% aluminum solution were added to a portion of each filtrate. A yellow color observed indicated the presence of flavonoids. When a portion of the powdered plant sample in each case was heated with 10 ml of ethyl acetate over a steam bath for 3 minutes and the mixture filtered and 4 ml of the filtrate shaken with 1 ml of dilute ammonia solution, a yellow coloration formed indicated a positive test for flavonoids.

#### 3.4.1.5 Test for steroids

Two milliliters of acetic anhydride was added to 0.5g ethanolic extract of each sample with 2 ml $H_2SO_4$. The color change from violet to blue or green in some samples indicated the presence of steroids.

#### 3.4.1.6 Test for terpenoids (Salkowski Test)

Five milliliters of each extract was mixed in 2 ml of chloroform, and concentrated $H_2SO_4$ (3ml) was added carefully to form a layer. A reddish brown coloration formed at the interface showed the presence of terpenoids.

### 3.5 Isolation of Endophytes from Plant Tissues

The medicinal plants selected for the study were collected from their natural habitats in Kakamega Forest. Disease free parts of the plants (stem and leaves) were cut into 5 cm pieces with a sterile scalpel and placed in zip-lock plastic bags materials stored at 4°C until isolation.

Endophytic isolation was carried out under aseptic conditions. Symptomless parts of the stem and the leaves were used for the isolation of the endophytes following the method of Santos *et al* ( 2003).Appropriate controls were also set up in which no plant tissues were inoculated. The purified endophytic isolates were transferred separately to Potato Dextrose Agar (PDA)

supplemented with chloramphenicol (100 mg/ml) and Nutrient Agar (NA) slants respectively and accessioned accordingly depending upon the plant parts from which they were isolated.

### 3.6 Identification of Endophytic Fungi and Bacteria

#### 3.6.1 Identification of endophytic fungi

The fungi were identified on the basis of colony characteristics, morphology and arrangements of spores on the fungal hyphae by using slide culture technique (Aneja, 2014)**.**

#### 3.6.2 Identification of endophytic bacteria

The isolated endophytic bacteria strains were identified by colony morphology, biochemical and physiological characteristics. In morphological characterization, macroscopic and microscopic features of the selected isolates were studied, additionally, an array of biochemical and physiological tests including catalase test, oxidase test, starch utilization test, nitrate reduction test, motility test, $H_2S$ production test, and sugar utilization test were performed following the test described by (Maregesi *et al.* 2008). The choice of these tests was on the basis of the gram reactions and guided by the classification of the according to Bergey's Manual of Determinative Bacteriology ((Torreblanca *et al.* 1986)

##### 3.6.2.1 Catalase test

Catalase is an enzyme, which is produced by microorganisms that live in oxygenated environments to neutralize toxic forms of oxygen metabolites; $H_2O_2$. The catalase enzyme neutralizes the bactericidal effects of hydrogen peroxide and protects them. Anaerobes generally lack the catalase enzyme. Catalase mediates the breakdown of hydrogen peroxide ($H_2O_2$) into oxygen and water. To find out if a particular bacterial isolate was able to produce catalase enzyme, small inocula of bacterial isolate were mixed into hydrogen peroxide solution (3%) and

the rapid elaboration of oxygen bubbles was to occur. The lack of catalase was evident by a lack of or weak bubble production.

#### 3.6.2.2 Oxidase test

A drop of physiological saline was placed on each end of a slide and a portion of the isolated colony was emulsified using a wire loop to make a thick suspension. A drop of rabbit plasma was added to one of the suspensions and mixed gently by rocking the slide. The presence of clumping of the organisms within 10 seconds was positive for the coagulase enzyme.

#### 3.6.2.3 Starch utilization test

A fresh (16- to 18-hour) pure culture of test bacteria as an inoculation source was used. A single isolated colony was picked and streaked on the surface of the agar medium and plates were incubated for 24 to 48 hours or longer (3 to 5 days) (3) at 35 ± 2$^{o}$C in an incubator. After proper incubation, the surface of the agar was flooded with Gram's iodine solution. A blue-black zone surrounding the bacterial growth indicated starch hydrolysis (+) by the organism due to its production of the extracellular enzymes. The zone would start out yellow (from the iodine) and become progressively lighter yellow and then clear. The lack of a clear zone surrounding the growth indicated that starch was present and had not been hydrolyzed and the organism did not produce the extracellular enzymes.

#### 3.6.2.4 Nitrate reduction

The nitrate broth was inoculated with a heavy growth of test organism using aseptic technique and incubated at 37°C for 24-48 hours. One dropperful of sulphanilic acid and one dropperful of α-naphthylamine was added to each broth. At this point, a colour change to red indicates a positive nitrate reduction test. No colour change indicates the absence of nitrite. This can happen

either because nitrate was not reduced to nitrite, then nitrite was further reduced to some other molecule. If no red colour formed then proceed to the next step by adding a small amount of zinc (a toothpick full0 to each broth. Zinc catalyzes the reduction of nitrate to nitrite with a colour change to red indicating a negative nitrate reduction test because this means that nitrate must have been present and must have been reduced to nitrite and no colour change means that no nitrate was present; thus no colour change at this point is a positive result.

**3.6.2.5 Motility test**

A well-isolated colony was picked with a sterile needle and the medium was stabbed within 1 cm of the bottom of the tube by ensuring that the needle was in the same line it entered as it was removed from the medium. The culture was incubated at 35°C for 18 hours until growth was evident. A positive motility test was indicated by a red turbid area extending away from the line of inoculation. A negative test was indicated by red growth along the inoculation line but no further.

**3.6.2.6 Hydrogen sulphide production**

A tube of sterile peptone water was inoculated with the test organism and a lead paper strip was inserted in the neck of the bottle above the medium and stoppered well. The culture was incubated at 35°C and examined daily for a blackening of the lower part of the strip. Blackening indicated production of hydrogen sulfide.

**3.6.2.6 SugarUtilizationTest**

The phenol broth media in the test tube with the Durham tube inserted was aseptically inoculated with an 18-24 hour broth culture of the test organism using an inoculating loop. The tubes were then incubated at 37°C for 18-24 hours; with longer incubation hours to confirm the negative

results. A positive result was indicated by a yellow colour showing that enough acid products had been produced by fermentation of the sugar to lower the pH of 6.8 or less. A delayed fermentation reaction may produce an orange color. A negative reaction was indicated by reddish or pink colour. A reddish or pink color in a clear tube could indicate a false negative. Bubbles trapped within the Durham tube indicated the production of gas. Even a single bubble was significant and denotes evidence of gas production. No bubbles within the Durham tube indicated a non-gas-producing organism.

## 3.7 Preparation of Crude Endophytic Extracts

### 3.7.1 Extraction of fungal endophytic metabolites

Fungal endophytic cultures were grown at 28°C in potato dextrose broth (Oxoid) for 14 days with shaking at 110 rpm in a shaker incubator. After the fermentation process the fermented broth was harvested by centrifugation to separate the supernatant for solvent extraction process with ethyl acetate as a solvent. Equal volume of supernatant and ethyl acetate was pooled in a separating funnel and shaken vigorously and allowed to settle. Organic phase was separated and collected into a clean tube with the procedure repeated 3 times to pool the organic phase. Later the extracted solvent was flash evaporated and residue obtained. The extracts were then dissolved in 1ml dimethyl sulfoxide (DMSO) and kept at 4°C for further antimicrobial studies.

### 3. 7.2 Extraction of bacterial endophytic metabolites

The bacterial isolate was inoculated in an Erlenmeyer flask containing 5L of Tryptic soy broth and incubated for 3 to 4 days. The fermentation flask was incubated at 28°C and 110 rpm on a rotary shaker for 7 days. After fermentation the culture broth was filtered and the filtrates extracted three times with ethyl acetate. The organic phase was passed through a pad of

anhydrous sodium sulphate and evaporated to dryness. The yields of the extract were determined and recorded. The extract was then used to determine biological activity.

### 3.8 Antimicrobial assay of Plant Extracts and Crude Endophytic Metabolites

#### 3.8.1 Preparation of test sample

Stock solutions were prepared by dissolving both extract (100mg/ml) and pure analytical grade antibiotics (10mg/ml Ampicillin and 10mg Nystatin) in Dimethyl sulfoxide.

#### 3.8.2 Test microorganisms

The test isolates of *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli* and fungal isolates of *Candida albicans* were used. Human pathogenic ATCC reference strains of bacteria and fungi were also used to screen antimicrobial activity of *Candida albicans* (ATCC 90028), bacterial strains of *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 700603), *and Pseudomonas aeruginosa (*ATCC 27853 and *Staphylococcus aureus (*ATCC 259323*).* These were standard susceptible microorganisms to demonstrate any biological activity of the plants and endophytic extracts. The strains were a kind donation from the stock cultures of Microbiology Laboratory, Medical School, University of Nairobi.

#### 3.8.3 Antimicrobial Screening by Agar well-diffusion method

Agar well-diffusion method was used to determine the antimicrobial activity. Mueller Hinton agar (MHA) and Potato Dextrose Agar (PDA) plates were swabbed (using sterile cotton swabs) with an 8 hour old - broth culture of respective bacteria and fungi. Wells (6 mm diameter, 1 cm deep and about 2 cm apart) were made in each of the plates using sterile cork borer. Stock solution of each plant extract was prepared at a concentration of 100 mg/ml in different plant extracts of hexane, chloroform, ethanol, ethyl acetate and dimethyl sulfoxide (DMSO). 100 µl of

different concentrations of plant solvent extracts (100mg/ml of extract and 10mg/ml of Ampicillin or Nystatin) were added using sterile syringe into the wells and allowed to diffuse at room temperature for 2 hours. Control experiments comprising inocula without plant extract were set up. The plates were incubated at 37°C for 18-24 hours for bacterial pathogens and 28°C for 48 hours fungal pathogens. The diameter of the inhibition zone (mm) was measured and the activity index was also calculated. Triplicates were maintained and the experiment was repeated thrice, for each replicates the readings were taken in three different fixed directions and the average values were recorded for antimicrobial activity.

### 3.8.4 Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentrations (MIC) were performed by a serial dilution technique using sterile microcentrifuge tubes. The different plant extracts of hexane, chloroform, ethanol, ethyl acetate and dimethyl sulfoxide (DMSO) were taken (100 mg/ml) and serial dilution of the extract with Mueller Hinton broth for bacterial and Potato Dextrose broth medium for fungal culture. The tubes were incubated for 72 hours at 37°C and 28$^{o}$C for bacteria and fungi respectively. The lowest concentrations without visible growth (at the binocular microscope) was noted and defined as MICs.

### 3.8.5 Determination of Minimum Bactericidal Concentration (MBC)

Mueller Hinton agar plates were prepared and those concentrations which showed the highest inhibition were swabbed on agar plates and incubated at 37°C for 24 hours. After 24 hours of incubation, results were observed and the number of bacterial colonies was counted.

### 3.9 Data Analysis

#### 3.9.1 Antimicrobial Activity Assay of the Extracts

A comparison of the antimicrobial activity of the samples with standard antibiotics was evaluated by applying a one way Anova statistical test. All values were expressed as the mean (SEM) and $P \leq 0.05$ values for any statistically significant differences. Numerical data was analyzed using the Kruskal-Wallis, non-parametric test using Graphpad™ Version 5 for Windows (Graphpad™ Software Inc., San Diego, California).

# CHAPTER FOUR

# RESULTS

## 4.1 Screening the plants for phytochemicals

The results of the qualitative phytochemical analysis of *Vernonia adoensis* and *Ocimum kilimandscharicum* are presented in Table 1. The results showed the presence of alkaloids, terpenoids, tannins, steroids, saponins and phenols in the two plants leaves and stems. Alkalloids were mainly in the chloroform extracts while terpenoids and phenols were predominantly in the chloroform and ethanol extracts of both plants. Steroids and saponins were only present in the ethanol solvent extracts. There were no flavonoids and glycosides in any of the two plants' parts in the solvents used. Chloroform and ethanol extracts showed the highest phytoconstituents with hexane showing the least in both plants. Terpenoids and phenols were the most dominant phytoconstituents extracts in all the solvents used as compared to alkaloids, tannins, steroids and saponins. There were more phytochemical substances in the *Ocimum kilimandscharicum* leaves as compared to the *Vernonia adoensis* leaves and the opposite was observed for the stem.

**Table 1: Photochemical screening of the *Ocimum kilimandscharicum* and *Vernonia adoensis* medicinal plants**

| | ***Ocimum kilimandscharicum*** | | | | | | ***Vernonia adoensis*** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Phytochemicals | Leaf extracts | | | Stem Extracts | | | Leaf extracts | | | Stem Extracts | | |
| | Hex | Chlo | Eth | Hex | Chlo | Eth | Hex | Chlo | Eth | Hex | Chlo | Eth |
| Alkalloids | - | + | - | - | - | - | - | + | - | - | + | - |
| Flavonoids | - | - | - | - | - | - | - | - | - | - | - | - |
| Terpenoids | + | - | + | + | - | + | + | + | + | + | - | + |
| Tannins | - | - | + | - | - | + | - | - | + | - | - | + |
| Glycosides | - | - | - | - | - | - | - | - | - | - | - | - |
| Steroids | - | - | + | - | - | + | - | - | - | - | - | + |
| Saponins | - | - | + | - | - | + | - | - | - | - | - | + |
| Phenols | - | + | + | - | + | + | - | + | + | - | + | + |

The table results show a qualitative photochemical analysis of *Vernonia adoensis* and *Ocimum kilimandscharicum* medicinal plants of the Kakamega Forest, Kenya. They were crude extracts of Hexane, Chloroform and Ethanol solvents obtained by a Soxhlation extraction method (Buchi). Key: Hex: Hexane; Chlo: Chloroform, Eth: Ethanol, +: Presence of phytochemical -: Absence of phytochemical

### 4.2 Isolation and screening of the metabolites from the endophytes of the *Vernonia adoensis* and *Ocimum kilimandscharicum*

#### 4.2.1 Isolation of the endophytes

Endophytes were isolated from the target plants as shown in Table 2. *Vernonia adoensis* leaves showed more culturable endophytes than the stems whereas *Ocimum kilimandscharicum* had more culturable endophytes in the stems than the leaves. The morphospecies of the isolated endophytes are as shown in Plate 3 (a, b, d and e) below. One isolate of the endophyte from both plants looked morphologically similar (See Plate 1 (b) and 2 (d). The bacterial endophytes in the *Vernonia adoensis* in the leaves and stems were moprphologically similar. One of the two bacterial endophytes in the *Ocimum kilimandscharicum* died on subculture and was not resurcitated in the subsequent subcultures.It was not immediately known why the organism died but this could be attributed to lack of optimal condtions in terms of nutrients and atmospheric requirements.

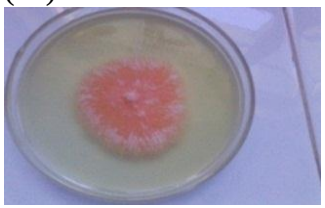
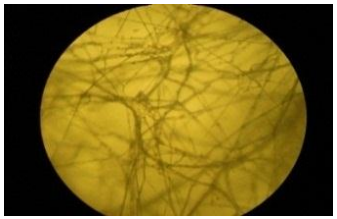
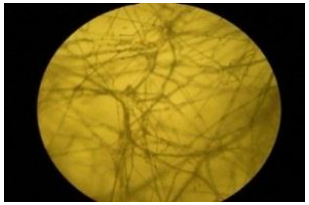
**Table 2: The isolated endophytes from the *Vernonia adoensis* and *Ocimum kilimandscharicum***

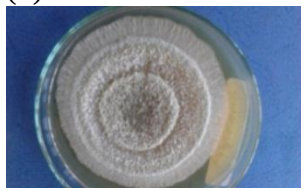
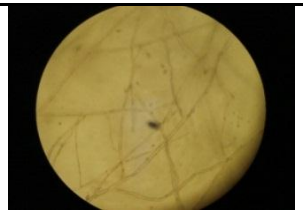
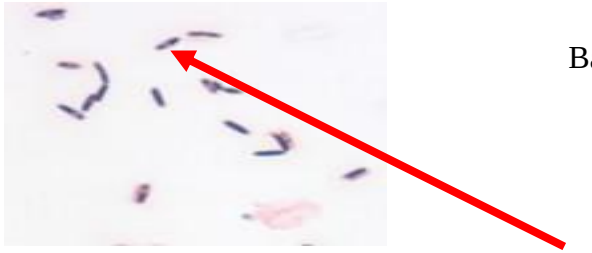
| Media | Vernonia adoensis | | Ocimum kilimandscharicum | |
|---|---|---|---|---|
| | Leaves | Stem | Leaves | Stem |
| PDA | *1 | 0 | 0 | 2 |
| NA | 1 | 1 | 1 | 2 |

The table shows the endophytes isolated from the plant parts (leaves and stems) of the *Vernonia adoensis* and *Ocimum kilimandscharicum* medicinal plants of the Kakamega Forest, Kenya. They were cultured on Potato Dextrose Agar (Fungi) and Nutrient Agar (Bacteria) media at 28°C. *Note: Number of endophyte isolated from the plant tissue

.

**Plate 1: Morphological and microscopic characteristics of the isolated endophytes from *Ocimum kilimandscharicum***

| Endophyte Isolate | Morphological Features | Microscopic Features | Taxonomic Placement |
|---|---|---|---|
| Fungal colony (BCF-1) | (a i)<br>  <br>3-4 days at 30°C<br>White, becoming pale brownish to pinkish, pale yellowish to pinkish colonies in reverse |  <br>Mycelium with highly branched non-septate globose mycelia. | Ascomycota (Alternaria sp) |
| | (a,ii)<br>  <br>White-pale brownish to pinkish, pale yellowish to pinkish colonies in reverse7-8 days at 30°C |  <br>Mycelium with highly branched non-septate globose mycelia. | Ascomycota (Alternaria sp) |

| | | | |
|---|---|---|---|
| Fungal colony (WCF-2) | (b)<br>  <br>Entire, circular sub-aerial hyphae with white concentric rings on PDA at 30°C |  <br>Mycelium with broad hyphae, septate. Chlamydospores numerous,<br>Thick walled intercalary or terminal, globose to subglobose or club-shaped, hyaline | Ascomycota (Phomopsis sp) |
| Bacterial colony (BaCB-1) | (c)<br>  <br>White, round with smooth and glossy surface as well as slightly opaque in nature |  | Bacillaceae)<br>Bacillus spp with spore |

The plates 1 and 2 show the morphological and microscopic characterization of the isolated endophytes from the *Ocimum kilimandscharicum* and *Vernonia adoensis* medicinal plants, respectively.

Microscopic characterization of the bacterial colonies isolates BaCB-1 (Plate 1) and BaCB-2 (Plate 2) revealed that both were gram positive rods with endospores. These strains were catalase, amylase, acetoin positive and they produced acid on glucose and mannitol. They also grew on media with 5.0 % NaCl. Based on these biochemical properties, the isolates were categorized as strains of *Bacillus* spp according to the Bergey's Manual of Systematic Bacteriology.

**Plate 2: Morphological and microscopic characteristics of the isolated endophytes from *Vernonia adoensis***

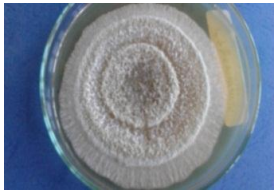
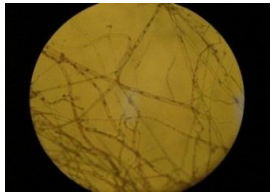
| Endophyte Isolate | Macroscopic Features | Microscopic Features | Taxonomic Placement |
|---|---|---|---|
| Fungal colony (WCF-3) | (d)  Entire, circular sub-aerial mycelia with dull- white concentric rings on PDA at 30°C |  Mycelium with broad hyphae, septate.Chlamydospores numerous, thick walled intercalary or terminal club-shaped, hyaline | Ascomycota (Phomopsis sp) |
| Bacterial Colony (BaCB-2) | (e)  White, round with smooth and glossy surface as well as slightly opaque in nature |  Gram-positive pleomorphic rods. Endospores were visible inside the cells in older cultures as clear areas inside the cells. | (Bacillaceae) Bacillus sp with spore |

**Plate 3: Morphological characteristics of bacterial endophytes from plants (BaCB-1) & (BaCB-2)**

| Tests | Result |
|---|---|
| Colony morphology |  White, round with smooth and glossy surface as well as slightly opaque in nature |
| Pigments | - |
| Grams reaction | + |
| Cell shape | Rods |
| Size | Long |
| Arrangement | Single |
| Spores | + (Endospore) |
| Motility | + |

The plate 3 shows the morphological and microscopic characterization of the isolated endophytes from the *Ocimum kilimandscharicum* and *Vernonia adoensis* medicinal plants. Key-**:** Negative for the test described +: Positive for the test described

**Table 3: Biochemical tests of the endophytic bacteria**

| S/NO. | Test Tests | Results |
|---|---|---|
| 1 | Gram stain | + |
| 2 | Oxidase | - |
| 3 | Indole Production | - |
| 4 | MR | + |
| 5 | VP | - |
| 6 | Citrate(Simmons) | - |
| 7 | Hydrogen sulfide production | - |
| 8 | Urea hydrolysis | + |
| 9 | Phenylalanine deaminase | - |
| 10 | Lysine decarboxylase | + |
| 11 | Motility | + |
| 12 | Gelatin hydrolysis, $22^{0}$ C | + |
| 13 | Acid from lactose | - |
| 14 | Acid from glucose | + |
| 15 | Acid from maltose | + |
| 16 | Acid from mannitol | + |
| 17 | Acid from sucrose | + |
| 18 | Nitrate reduction | + |
| 19 | Lipase | + (to nitrite) |
| 20 | Catalase production (24h) | + |

Biochemical tests carried out on the isolated endophytes from the *Ocimum kilimandshari*cum and *Vernonia adoensis* medicinal plants Key: +: Positive for the test described **-:** Negative for the test described;

**Table 4: Physiological characteristics of the bacterial endophytes**

| S/NO | Tests | Results |
|---|---|---|
| | Temperature | + |
| 1 | 25°C | + |
| 2 | 30°C | + |
| 3 | 37°C | + |
| 4 | 42°C | + |
| 5 | 45°C | + |
| | pH | |
| 1 | 5.0 | + |
| 2 | 6.0 | + |
| 3 | 7.0 | + |
| 4 | 8.0 | _ |
| 5 | 9.0 | _ |
| 6 | 12.0 | _ |
| | Growth on Nacl (%) | |
| 1 | 2.5% | + |
| 2 | 5.0% | + |
| 3 | 7.0% | _ |
| 4 | 10.0% | _ |

The test results in the table show the physiological properties of endophytes in terms of temperature, pH and their growth in varied concentrations of sodium chloride. Key: **-:** Negative for the test described +: Positive for the test described

### 4.2.2 Screening of the endophytic metabolites

Both fungal isolates WCF-2 and BCF-1 from *Ocimum kilimandscharicum* and BCF-3 from *Vernonia adoensis* were positive for flavonoids. Only the bacterial isolate (BaCB-1) from the *Vernonia adoensis* was positive for flavonoids. All the endophytes except the WCF-2 tested positive for alkalloids in both plants. WCF-2 in *Ocimum kilimandscharicum* and BCF-3 from *Vernonia adoensis* were positive for terpenoids. None of the isolates showed presence of glycosides, steroids, tannins, saponins and phenols (Table 5).

**Table 5: Screening of endophytic extracts from *Ocimum killimandscharicum* and *Vernonia adoensis.***

| Secondary metabolites | *Ocimum kilimandscharicum* | | | *Vernonia adoensis* | |
|---|---|---|---|---|---|
| | Bacteria BaCB-1 | White colony WCF-2 | Brown colony BCF-1 | Brown colony BCF-3 | Bacterial colony BaCB-2 |
| Alkalloids | + | - | + | + | + |
| Terpenoids | - | + | - | - | + |
| Flavonoids | + | + | + | + | - |
| Glycosides | - | - | - | - | - |
| Steroids | - | - | - | - | - |
| Tannins | - | - | - | - | - |
| Saponins | - | - | - | - | - |
| Phenols | - | - | - | - | - |

The table shows the qualitative analysis of the crude ethyl acetate extracts from the endophytes of the *Vernonia adoensis* medicinal plant. +/-: Presence/absence of the secondary metabolites from the endophyte

### 4.3 Antimicrobial Screening of the Crude extracts from Plants and Endophytes

#### 4.3.1 Antimicrobial activity of the phytochemical extracts of the plants

The results in this study showed that chloroform and ethanol extracts in both plants exhibited significant antimicrobial activity compared to the other extracts. The chloroform and ethanol extracts from both plants showed the highest activity against *Escherichia coli* and *Staphylococcus aureus.* Only the ethanol extract from the *Vernonia adoensis* showed a significant activity against *Candida albicans*. The hexane extracts from the two plants showed limited activity against *Klebsiella pneumoniae* and *Staphylococcus aureus* in both plants and only *Staphylococcus aureus* in *Vernonia adoensis.* There was no activity against *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* from the extracts of the *Ocimum kilimandscharicum* and limited activity of the Chloroform extracts against the *Klebsiella pneumoniae*.

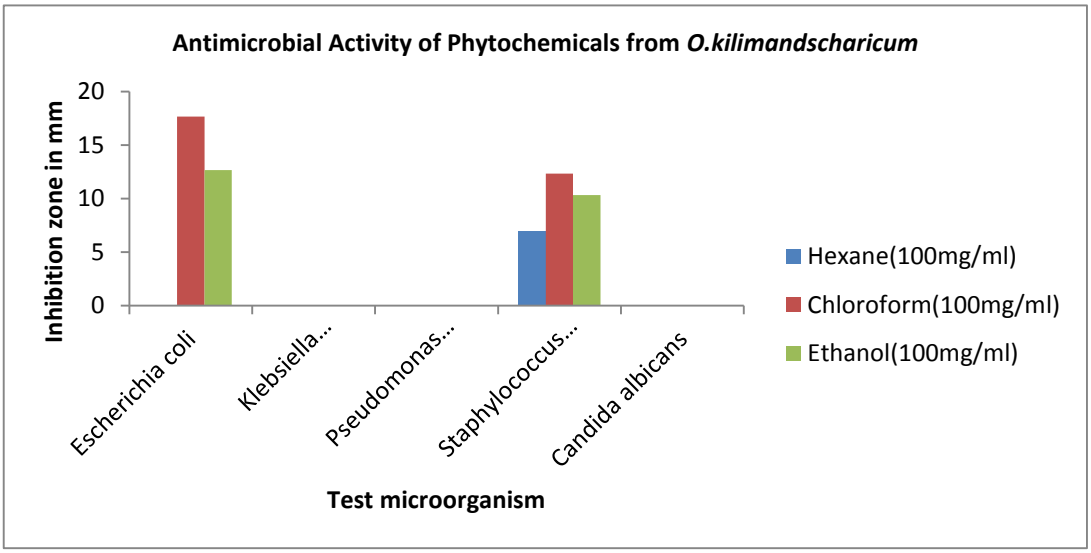



**Figure 1: Antimicrobial Activity of Crude Phytochemical Extracts from *Ocimum killimandscharicum***

This figure shows the antimicrobial activity of the crude extracts from the *Ocimum kilimandscharicum* in hexane, chloroform and ethanol solvents.

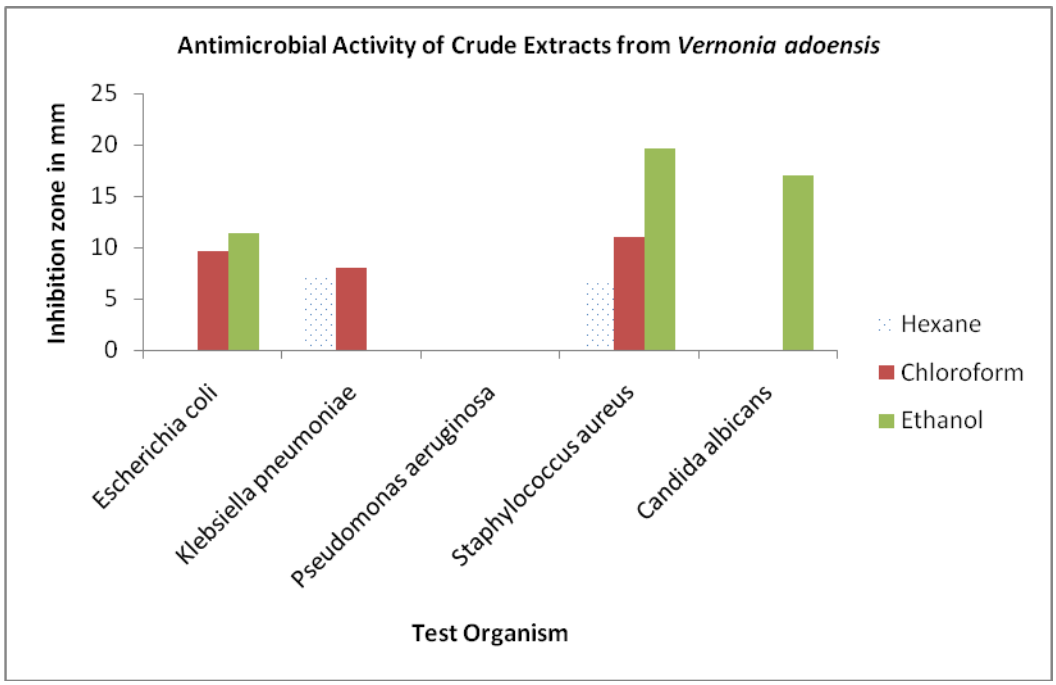



**Figure 2: Antimicrobial Activity of Crude Phytochemical Extracts from *Vernonia adoensis***

The figure shows the antimicrobial activity of the crude extracts from the *Vernonia adoensis* in hexane, chloroform and ethanol solvents.

**Table 6: Antimicrobial screening of crude phytochemical extracts from the plants**

| Test Organisms | Diameters of Zones of Inhibition in mm | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *Ocimum kilimandscharicum* | | | | *Vernonia adoensis* | | | | |
| | Hex | Chloro. | Ethanol | Control Amp/Nyst | Hex. | Chlo | Ethanol | Controls: Amp/Nyst. | p-values |
| ***E. coli*** | NI | 17.67± 0.33 | 12.67±0.33 | 21.00± 1.00 | NI | 9.67± 0.33 | 11.33±0.33 | 23.67± 0.33 | 0.0138 |
| ***K. pneumoniae*** | NI | NI | NI | 14.00 ± 0.577 | 7.33± 0.33 | 8.00± 0.00 | NI | 19.00± 0.00 | |
| ***Ps. aeruginosa*** | NI | NI | NI | 13.67± 0.33 | NI | NI | NI | 13.67± 0.33 | |
| ***S. aureus*** | 7.00± 0.00 | 12.33 ±0.33 | 10.33± 0.33 | 19.67 ± 0.33 | 6.67±0.33 | 11.00±0.77 | 19.67±0.67 | 20.33± 0.88 | 0.0223 |
| ***C. albicans*** | NI | NI | NI | 17.00 ± 0.577 | NI | NI | 17.00±0.577 | 17.00±0.577 | |

Antimicrobial Analysis of Crude Phytochemical Extracts from the *Ocimum kilimandscharicum* and *Vernonia adoensis* medicinal plants in different solvents. Diameter of inhibition zone (mm±SEM, n=3) for pathogenic bacteria and fungi
Key: Hex. – Hexane, Chloro. - Chloroform, Eth. – Ethanol, Amp/Nyst- Ampicillin/Nystatin
NI: No inhibition at the concentration used. Note that the concentrations used: Extracts: 100mg/ml and 10mg/ml for Ampicillin/Nystatin.

### 4.3.2 Antimicrobial activity of the endophytic crude ethyl acetate extracts

Screening of the endophytic fungi and bacteria to determine the antimicrobial activity was done by agar well diffusion method against five pathogenic human strains after 21 days old metabolites of the endophytes. The bacteria provided maximum zone of inhibition after 72 hours while the fungi (*Candidaalbicans*) took seven days. WCF-1 from *Ocimum* showed maximum zone of inhibition against *Candidaalbicans* (20 mm) but least activity to *Klebsiella pneumoniae.* BCF-1had limited activity against *Escherichia .coli, Klebsiella pneumoniae and Candida albicans.* BCF-2 from *Vernonia adoensis* had moderate activity while BaCB-2 had strong activity against *K.pneumoniae.* There was no activity of the BCF-2 and BaCB-2 extracts from *Vernonia adoensis* against *Ps. aeruginosa, S.aureus* and *C.albicans*.

**Table 7: Antimicrobial screening of the endophytic extracts**

| **Test Organism** | **Diameters of zones of inhibition in mm** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *Ocimum kilimandscharicum* | | | | *Vernonia adoensis* | | | |
| | BCF-1 | WCF-1 | BaCB-1 | Control; Amp/Nyst | BCF-2 | BaCB-2 | Control; Amp/Nyst | p-values |
| ***E. coli*** | 7.33± 0.33 | NI | NI | 23.00±0.577 | 12.33±0.33 | NI | 25.33± 0.33 | 0.0226 |
| ***K. pneum*** | 10.33±0.33 | 10.33±0.33 | NI | 15.33±0.33 | NI | 15.33±0.33 | 15.33±0.33 | 0.0211 |
| ***Ps. aerug.*** | NI | NI | NI | 14.00± 0.00 | NI | NI | 14.00± 0.00 | |
| ***S. aureus*** | NI | NI | NI | 21.33±0.33 | NI | NI | 21.33± 0.67 | |
| ***C.albicans*** | 10.67±0.33 | 20.33±0.33 | NI | 12.00±0.577 | NI | NI | 12.00±0.577 | 0.0001 |

The table shows the antimicrobial activity of the endophytic extracts in ethyl acetate against the standard pathogenic bacteria and fungi. Zones of inhibition were measured in millimeters (mm) and the standard error of the median (SEM) for each test stated. Diameter of inhibition zone (median±SEM, n=3) for pathogenic bacteria and fungi. Key: NI: No inhibition.

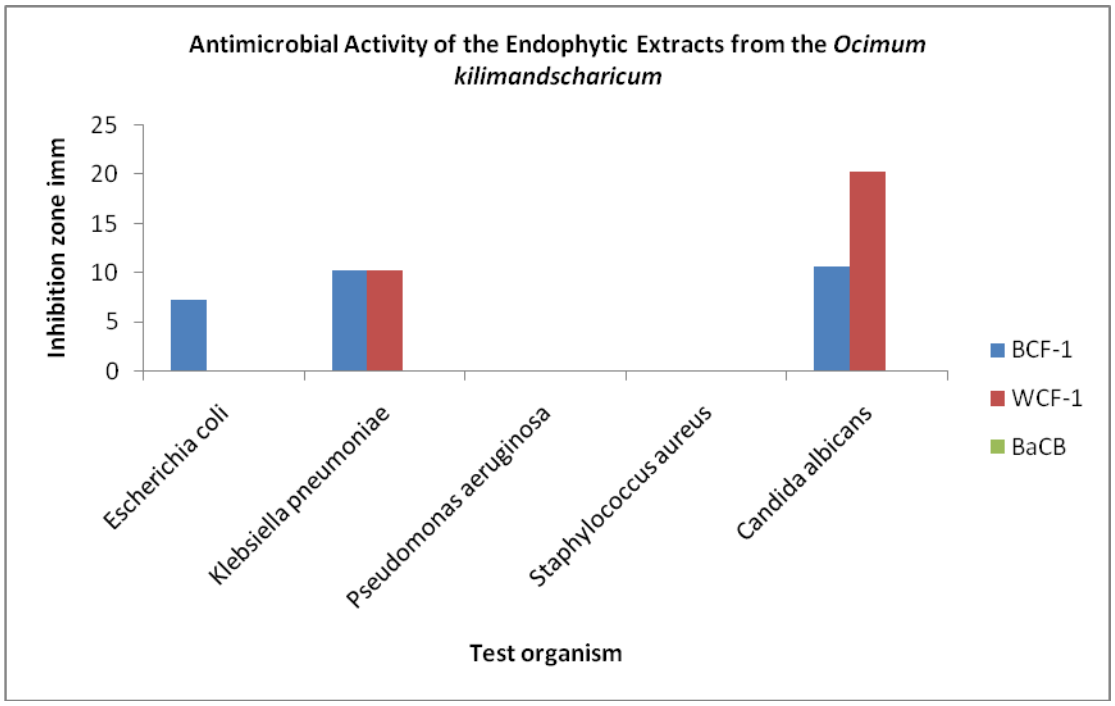



**Figure 3: Antimicrobial Activity of the Endophytic Extracts from the *Ocimum kilimandscharicum***

The figure shows the antimicrobial activity of the endophytic extracts (BCF-1, WCF-1, and BaCB) from the *Ocimum kilimandscharicum* medicinal plant against standard bacteria and fungi. Zones of inhibition were measured in millimeters (mm).

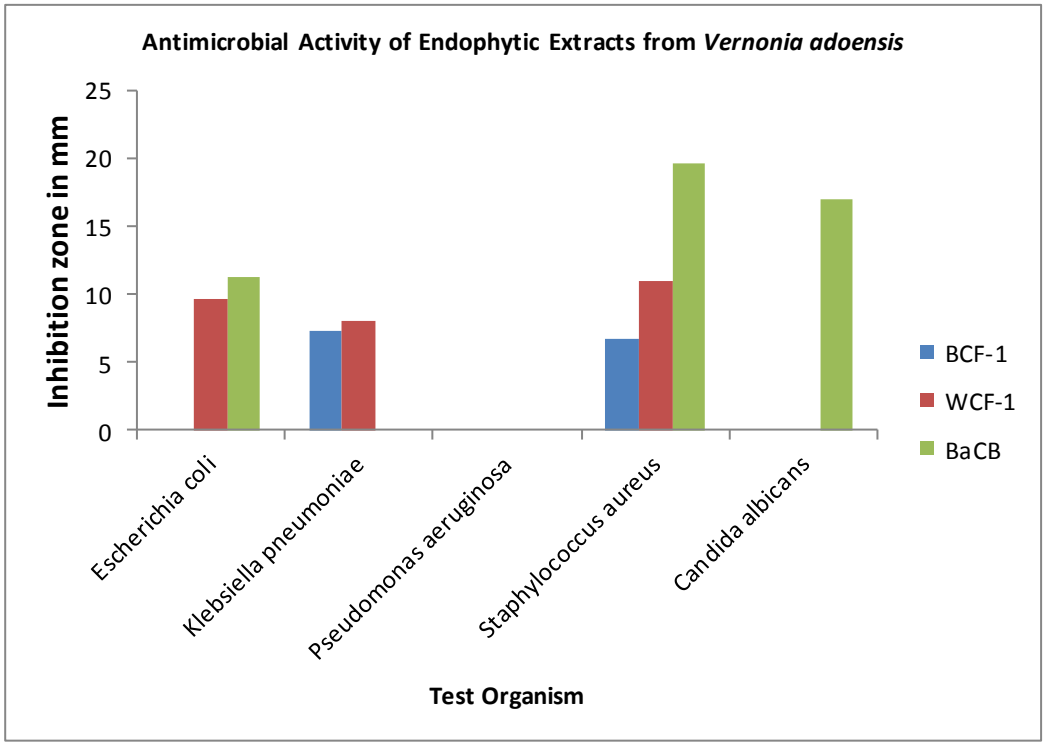



**Figure 4: Antimicrobial Activity of the endophytic extracts of *Vernonia adoensis* in ethyl acetate**

The figure shows the antimicrobial activity of the endophytic extracts in ethyl acetate from the *Vernonia adoensis* medicinal plant against clinical cases of bacteria and fungi

### 4.4 Determination of Minimum Inhibitory Concentration

#### 4.4.1 Minimum Inhibitory Concentration of the crude phytochemical extracts

Determination of MIC is important in diagnostic laboratories because it helps in confirming resistance of microorganisms to an antimicrobial agent and it monitors the activity of new antimicrobial agents. In this study, antimicrobial activities of the phytochemical and endophytic extracts were carried out as shown in table 8 below. The most active extracts against the tested microorganism in the *Ocimum* and *Vernonia* plants were the chloroform and ethanol with 12.5

mg/ml and 25.00mg/ml each respectively against *Staphylococcus aureus*. *Escherichia coli* was inhibited by the chloroform and Ethanol extracts at 25.00mg/ml and 50.00mg/ml in *Ocimum* and *Vernonia* plants respectively. No inhibition was detected in *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and the *Candida albicans* for all the other extracts in the two plants.

**Table 8: Minimum Inhibitory Concentration (MIC) of extracts from plants (phytochemicals)**

| Test Organisms | MIC (Concentrations (mg/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *Ocimum kilimandscharicum* | | | | *Vernonia adoensis* | | | |
| | Hex | Chloro. | Ethanol | Control Amp/Nyst | Hex | Chloro. | Ethanol | Control: Amp/Nys |
| *E. coli* | NI | 25.00 | 25.00 | 2.25 | NI | 50.00 | 50.00 | 5.00 |
| *K. pneum* | NI | NI | NI | 2.25 | NI | NI | NI | 2.25 |
| *Ps. aerug* | NI | NI | NI | 2.25 | NI | NI | NI | 5.00 |
| *S. aureus* | NI | 12.5 | 12.5 | 1.25 | NI | 25.00 | 25.00 | 1.25 |
| *C.albicans* | NI | NI | NI | 5.00 | NI | NI | NI | 5.00 |

The results in the table show the MIC of the crude extracts of the medicinal plants in the hexane, chloroform and ethanol solvents. NI-: No inhibition

### 4.4.2 Minimum Inhibitory Concentration of the crude endophytic extracts

The BCF-1 and BaCB-1 in*Ocimum kilimandscharicum* and BCF-2 and BaCB-2 from *Vernonia* ethyl acetate extracts inhibited *Klebsiella pneumoniae* at 50.00mg/ml and 25.00mg/ml MICs respectively, with 25.00mg/ml of WCF-1 being inhibitive to *Candida albicans* and BCF-2 with 50.00mg/ml against *Candida albicans* from *Vernonia adoensis.* The most resistant of the five microorganisms were *Pseudomonas aeruginosa* and *Staphylococcus aureus*against all the extracts from both plants. *Klebsiella pneumoniae* was sensitive to most of the extracts with

*Candida albicans* inhibited byBCF-1, WCF-2(both at 25mg/ml) from *Ocimum kilimandscharicum* and BCF-2(50mg/ml) extracts from *Vernonia adoensis.* The most inhibitive extract was that of WCF-1 and BCF-1 against *Klebsiella pneumoniae* from *Ocimum kilimandscharicum* and BaCB-2 against from *Vernonia adoensis* against *Klebsiella pneumoniae.*

**Table 9: Minimum Inhibitory Concentration (MIC) of crude (ethyl acetate) extracts from endophytes**

| Test Organisms | MIC (Concentrations (mg/ml) | | | | | | |
|---|---|---|---|---|---|---|---|
| | *Ocimum kilimandscharicum* | | | | *Vernonia adoensis* | | |
| | BCF-1 | WCF-1 | BaCB-1 | Control; Amp/Nyst | BCF-2 | BaCB-2 | Control; Amp/Nyst |
| *E. coli* | NI | NI | NI | 5.00 | 25.00 | NI | 5.00 |
| *K. pneum.* | 50.00 | NI | 50.00 | 2.25 | 25.00 | 25.00± | 2.25 |
| *Ps. aerug.* | NI | NI | NI | 5.00 | NI | NI | 5.00 |
| *S. aureus* | NI | NI | NI | 1.25 | NI | NI | 1.25 |
| *C. albicans* | 25.00 | 25.00 | NI | 5.00 | 50.00 | NI | 5.00 |

The values shown are mg/ml concentration of endophytic extracts against 5 indicator strains, NI: No inhibition. MIC- (mg/ml), n=3) for pathogenic bacteria and fungi

### 4.5 Determination of Minimum Bactericidal/Fungicidal Concentration (MBC/MFC)

#### 4.5.1 Minimum Bactericidal/Fungicidal Concentration of the plant phytochemicals

The plants crude extract concentrations which had shown the highest inhibition/MIC were swabbed on Mueller Hinton agar and Potato Dextrose Agar plates and incubated at 37°C and 28°C respectively. After 24 hours of incubation, results of the MBC & MFC were as in table 4.34 below;

**Table 10: MBC/MFC of extracts from plants (phytochemicals)**

| Test Organisms | MBC/MFC-Concentrations (mg/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *Ocimum kilimandscharicum* | | | | *Vernonia adoensis* | | | |
| | Hex | Chlo | Eth | Control Amp/Ny | Hex | Chlo | Eth | Controls Amp/Ny |
| *E. coli* | NI | 50.00 | 50.00 | 5.00 | NI | 100.00 | 100.00 | 5.00 |
| *K. pneum* | NI | NI | NI | 5.00 | NI | NI | NI | 2.25 |
| *Ps. aerugin* | NI | NI | NI | 5.00 | NI | NI | NI | 5.00 |
| *S. aureus* | NI | 25.00 | 12.5 | 1.25 | NI | 100.00 | 100.00 | 2,50 |
| *C. albicans* | NI | NI | NI | 10.00 | NI | NI | NI | 10.00 |

The values shown in are mg/ml concentration of extracts against 5 indicator strains. Key: NI: No inhibition. MBC/MFC- (mg/ml), n=3) for pathogenic bacteria and fungi;

The chloroform and ethanol extracts from both plants had significant MBCs for *Escherichia coli* and *Staphylococcus aureus* respectively. The rest of the extracts did not inhibit any other pathogenic microorganisms tested.

### 4.5.2 Minimum Bactericidal/Fungicidal Concentration of the endophytic extracts in ethyl acetate

The MBC for *Klebsiella pneumoniae* against BCF-1 and BaCB-1 from the *Ocimum kilimandscharicum* was 100.00mg/ml each and 50.00mg/ml each for BCF-2 and BaCB-1 metabolites from the *Vernonia adoensis*. The lowest MBC/MFC was recorded in BCF-1 and WCF-2 for *Candida albicans* from *Ocimum kilimandscharicum* and BCF-3 and BaCB-1 against *Klebsiella pneumoniae* and *Candida albicans* extracts from the *Vernonia adoensis*.

**Table 11: MBC/MFC of crude (ethyl acetate) extracts from endophytes**

| Test Organisms | MBC/MFC-Concentrations (mg/ml) | | | | | | |
|---|---|---|---|---|---|---|---|
| | *Ocimum kilimandscharicum* | | | | *Vernonia adoensis* | | |
| | BCF-1 | WCF-2 | BaCB-1 | Control; Amp/Nys | BCF-3 | BaCB-1 | Control; Amp/Ny |
| *E. coli* | NI | NI | | 10.00 | NI | | 10.00 |
| *K. pneum* | 100.00 | NI | 100.00 | 5.00 | 50.00 | 50.00 | 5.00 |
| *Ps. aerugin* | NI | NI | NI | 10.00 | NI | NI | 10.00 |
| *S. aureus* | NI | NI | NI | 2.50 | NI | NI | 5.00 |
| *Candida alb* | 50.00 | 50.00 | NI | 10.00 | 50.00 | NI | 10.00 |

The values shown are mg/ml in concentration of extracts against 5 indicator strains. MBC/MFC-(mg/ml), n=3) for pathogenic bacteria and fungi. Key: NI: No inhibition.

# CHAPTER FIVE

# DISCUSSION

This study showed how the phytochemicals in the medicinal plants are related to those from the endophytes from the tissues of these plants. It also shows that the secondary metabolites from the plants and the endophytes have a significant antimicrobial activity against bacteria and fungi. Taken together these data showed that the metabolites from the plants and the endophytes are potential antimicrobials and justifies the reason for their use in traditional medicine.

## 5.1 Phytochemical screening of crude extracts from the *Vernonia adoensis* and *Ocimum kilimandscharicum*

Medicinal plants contain many secondary metabolites that are found to be responsible for several biological activities in human beings and animals (Sofowora, 1982). A significant number of studies that have been done on a number of plants to obtain purified plant chemicals indicate that the medicinal value of plants lies in the phytoconstituents present in those plants (De Filippis, 2016; Evans *et al.*,2006). Crude extracts of medicinal plants belonging to *Asteraceae* (*Vernonia spp*) and *Lamiaceae* (*Ocimum basilicum*) showed a significant antimicrobial activity against resistant bacteria and fungi(Amuka 2014). Incidentally, very few screening programmes have been initiated on crude plant materials on many of the local medicinal plants commonly used in the traditional medicine.

In this investigation, the active phytocomponents of *Vernonia adoensis* and *Ocimum kilimandscharicum* were analysed and studied. The screening of these phytoconstituents in both plants revealed that they contained alkalloids, terpenoids, tannins, steroids, saponins and phenols in the solvents used. Flavonoids and glycosides were absent in all the solvents used in this study. It has been suggested that the distribution of these phytoconstituents in the plant is dependent on

the age of the plant at the time of sample collection, its geographical location (Khattak *et al.*, 2015)and the handling and processing (Dhanani *et al*. 2016) of the plants during the extractions. The absence of the flavonoids in the ethanol extracts in this study contrasts with the findings of Swamy (2013) that contained flavonoids. Palermo *et al.*, (2014) also showed that ethanol/methanol give very positive isolates of flavonoids when percolation method of extraction is used. It is possible that heating (soxhlation) method as was used in this study tends to denature most flavonoids and other oxidants despite the fact that it increases the extractability of phytochemicals. Therefore, the absence of flavonoids and glycosides may be partially due to the extraction method used in this study. Other factors that may affect the presence of the phytochemicals in the extracts are the type of solvent, the treatment of the plant sample prior to extraction and the weight (amount) of the plant sample to be extracted among other factors as mentioned by Khattak *et al.*,(2015).

Phenolic compounds and tannins are the major group of compounds acting as primary antioxidants or free radical scavengers. Tannins are known to possess general antimicrobial and antioxidant activities. Recent reports show that tannins may have potential value as cytotoxic and antineoplastic agents (Sowjanya *et al.* 2013).Saponins found in the plants have no known physiological roles, though they are found to have a broad spectrum antimicrobial activity against bacteria, fungi and viruses (Naidu, 2000). The presence of saponins in this study shows the potential of the plants to be utilized as a source of antibiotics as suggested by earlier studies Jackie *et al.* (2014).

Alkaloids are secondary metabolites or cyclic compounds that have nitrogen in negative oxidation state and usually have an effect on the chemical transmitters of the nervous system (Chikezie *et al.*, 2015). They also have other pharmacological activities such as analgesic,

antispasmodic, antihypertensive effects and antiarrhymic effects and antibacterial (Saxena *et al*. 2013). According to Karou *et al.(*2006) alkaloids have good antibacterial activity with highest zone of inhibition recorded on gram-positive bacteria. Cryptolepine a major alkaloid in *S.acuta* was found to be an antimalarial agent (Banzouzi *et al.* 2004). Cryptolepine has also been used clinically to treat malaria, colic and stomach ulcers and also used as anticancer drugs (Bonjean *et al.* 1998). According to Karou *et al.* (2006) have been done on pharmacological properties of alkaloids on antiprotozoal, cytotoxic and anti-inflammatory properties. The findings in this research are in agreement with those of other similar researches in the past and demonstrate a very significant activity against *Klebsiella pneumoniae* and *Escherichia coli*.

Steroids, have been reported to have antibacterial activity (Epand *et al*., 2007). Besides this they are very important class of compounds in particular due to their relationship with compounds such as sex hormones (Singh *et al.* 2007). Findings in this study showed no activity of the steroids to any bacteria tested.

The phenolic compounds possess different biological activities such as anti-apoptosis, antiaging, anticarcinogen, anti-inflammatory, ant atherosclerosis (Han *et al.*, 2007) and the more recent cardiovascular protection and improvement of endothelial function, as well as inhibition of angiogenesis and cell proliferation activities has also been reported (Brown *et al.*, 1998). The antioxidant activity of the various plants rich in phenolics have also been described by various workers (Molan *et al.*, 2016). From this study it is evident that the plants possessed pharmacologically active phytoconstituents and justifies their use in ethnomedicine.

### 5.2 Endophytic isolation and fermentation to produce bioactive metabolites

In traditional medicine, plants are assumed to have some healing power that may be due to unknown bioactive compounds ( Strobel *et al.*,2003). In this study, *Vernonia adoensis* and *Ocimum kilimandscharicum* were selected for the isolation of endophytic bacteria and fungi based on their medicinal importance as reported by the local people.

Two species of endophytic fungi and one species of endophytic bacteria from stems and leaves of *Vernonia adoensis* and *Ocimum kilimandscharicum* were isolated and identified, belonging to genus *Alternaria* and *Phomorpsis* for fungi and *Bacillus* for bacteria**.**

Research reports indicate that endophytic microbes from various plants exist in different ecosystems (Strobel *et al.,* 2003). Nearly 300 000 plant species that exist on earth, each individual plant is host to one or more endophytes (Rajendran, 2016). Incidentally, a few of these plants have been completely studied relative to their endophytic biology. Consequently, the opportunity to find new and interesting endophytic microorganisms among myriads of plants in different settings and ecosystems is great (Strobel *et al.*, 2003). Kumar *et al.,* (2015) showed that species composition and frequency of endophyte species are dependent on the tissue type of the host plant. Raviraja (2005) also reported on the fungal endophytes in five medicinal plant species from India. The fungal endophytes were found to be host and tissue specific which is similar to the findings of this study. In another study, Gangadevi *et al.,* (2007) studied endophytic fungal diversity in young, old and senescent leaves of *Ocimum basilicum* L. medicinal plant and found that the isolates of *Phyllosticta* sp. produced taxol in artificial culture media which is an anticancer agent. There is no information on the endophytes of the *Vernonia adoensis* and *Ocimum kilimandscharicum.*

In the current study, the isolated endophytes were fermented and the metabolites were extracted using ethyl acetate solvent. The phytochemical analysis of the metabolites revealed the presence of alkalloids, terpenoids and flavonoids. There were no cardiac glycosides, steroids, tannins, saponins and phenols of the endophyte extracts from both plants. The ability of an endophyte to produce some metabolites but not others was also described by Digrak *et al.* (1999) where different endophytes in a plant may produce different secondary metabolites. Different metabolites play different functions in the plant and that the total number of metabolites in a plant extract may be a contribution of all the endophytes that live in the plant. The production and quality of bioactive compounds from endophytic fungi depends on natural conditions of the association and the nature of the synthetic medium used. Sudhakar *et al.* (2013) hypothesized that endophytic fungi possess pathway genes for plant secondary metabolites and states that the secondary metabolites produced by the fungi while in the plant are also produced by the host plant at same time. The endophyte fails to produce the same secondary metabolite if cultured outside the plant suggesting that the endophyte lacks a host stimulus in the culture media and/or silencing of genes in axenic cultures to produce the same metabolite. This line of thought prompted Tan *et al.*,(2001b) to suggest that genetic recombination of the endophyte with its host in evolutionary time could have led to the incorporation of the pathway genes of the host into the endophyte. However, there is no proof for the horizontal transfer of genes coding for secondary metabolites between a plant host and its endophytic fungal associate. In effect, the mechanism underlying the production of plant secondary metabolites by an endophytic fungus remains enigmatic. The production of some of the secondary metabolites from the endophytes in this study agrees with the above hypothesis and arguments supported by Tan *et al.*,(2001b). It is however, not clear from the study findings whether the secondary metabolites produced in this

study could be more if the stimulus was present and also if those produced are structurally similar to those produced by the plant in the same conditions and ecological environments/settings.

Likewise, bacterial and fungal endophytes have been reported from various medicinal plants; for examples, *Piper nigrum, Gynura procumbens, Strobilanthes crispa, Vernonia amygdalina* and Aquilaria species (Aravind *et al.* 2009; Subhash *et al*.,2010 and Bhore *et al.*, 2012). Endophytic bacteria have also been isolated from *Ocimum sanctum* (Tiwari *et al.* 2010) and *Penicillium janczewskii from Vernonia herbacea* (Asteraceae). In this study it was the first one to be identified and isolated from the *Vernonia adoensis* and *Ocimum kilimandscharicum* from the Kakamega forest with a unique ecological complexity compared to those other environments so far studied.

### 5.3 The Antimicrobial Activity of the Crude Extracts from the Plants

#### 5.31 Antimicrobial screening of crude extracts from the plants

The antimicrobial results showed that the chloroform (17.67± 0.33mm) and ethanol (12.67±0.33mm) extracts exhibited an antimicrobial activity in *Ocimum kilimandscharicum* and 9.67± 0.33mm and 11.33±0.33mm chloroform and ethanol extracts respectively in *Vernonia adoensis* against the *Escherichia coli. S.aureus* was moderately sensitive to the chloroform and ethanol extracts showing 12.33 ±0.33 mm and 10.33± 0.33mm respectively in *Ocimum kilimandscharicum* and 11.00±0.577 mm and19.67± 0.67mm respectively from *Vernonia adoensis*. There was no observable inhibition of all the extracts from the *Ocimum kilimandscharicum* and *Vernonia adoensis* against the *Candida albicans* except ethanol extract from the *Vernonia adoensis* that showed an inhibition zone of 17.00±0.577mm. The hexane extracts exhibited weak activity against *Staphylococcus aureus (*7.00± 0.00mm) and

(6.67±0.33mm) in both plants but *Klebsiella pneumoniae* in *Vernonia adoensis*. *Pseudomonas aeruginosa* did not respond to any of the extracts tested**.** The hexane extracts exhibited the least antimicrobial activity as compared to other three solvent extracts. The reasons for minimal antibacterial activity in hexane extracts could be a low concentration of antimicrobial compounds in these extracts. This study is in agreement with other investigators in the past which showed that ethanol was the most effective solvent for extracting phytochemicals from plants (Dahiya *et al.*,, 2016).These results also agree with how in the traditional medicine, the natives used local alcohol brews to take herbal medicines that were found to be useful in the management of various illnesses (Iversen,2016 and Odugbemi, 2008)**.**

These results showed that the most sensitive tested microorganisms to the extracts from the two plants were *Staphylococcus aureus* and *Escherichia coli.* In similar studies in the past, most of the studies were directed to the activity of plant extracts against a variety of test bacteria and fungi including both pathogenic and nonpathogenic strains. Several studies have made targeted screening against MDR bacteria such as MRSA, VRE, *M. tuberculosis*, enteric bacteria and others (Dahiya *et al.*,2012a). A study by Dahiya *et al.,* (2012b) showed that the most susceptible organism was *S. aureus* ATCC 25923 which was sensitive to 29 plant extracts in various solvents except for hexane extract of lemongrass and chloroform extracts of both lemongrass and bryophyllum. *S. aureus* 1 was sensitive to 24 extracts while *S. aureus* 2 was sensitive to 21 extracts. Findings from this study favorably shares with the investigations of Dahiya (2016) that plant solvent extracts have antimicrobial activity to bacterial microorganisms that. In another study by (Toyang *et al.,* 2013) showed that *Vernonia amygdalina* was the most frequently used member of the *Vernonia* genus and found that the plant possess a Vernolide which had an a substantial antimicrobial activity against the clinical cases of microbial strains of bacteria and

fungi. The antimicrobial activity exhibited by the chloroform and ethanol extracts from the *Vernonia adoensis* against the *Escherichia coli* and *Staphylococcus aureus* in this study may be compared to the findings of Toyang *et al*. (2013 ) and is indicative of the plants possessing an antimicrobial agent.

Various studies have already shown that Gram positive bacteria are more susceptible towards plants extracts as compared to Gram negative bacteria (Parekh *et al.,* 2007). These differences may be attributed to fact that the cell wall in Gram positive bacteria is of a single layer, whereas the Gram negative cell wall is multilayered structure (Yao *et al.* 1999). The observed differences may also result from the doses used in this study. In addition, microorganisms show variable sensitivity to chemical substances related to different resistance levels between strains. It is worth noting that the differences in the antimicrobial effects of plant groups may be due to phytochemical properties and differences. This may be partially be attributed to different plant metabolites that work in combination with other compounds to regulate microbial infections and may therefore not be effective alone (Lewis *et al.*, 2006)

In the investigated plants the hexane extracts did not show strong antimicrobial activity. Active compound(s) may be present in insufficient quantities in the crude extracts to show activity with the dose levels employed (Taylor *et al.* 2001). Lack of activity can thus only be proven by using large doses (Farnsworth, 1993). Alternatively, if the active principle is present in high enough quantities, there could be other constituents exerting antagonistic effects or negating the positive effects of the bioactive agents. With no antibacterial activity, extracts may be active against other bacterial species which were not tested as suggested by Shale *et al*.,(1999)

In these plants species investigated, ethanol extracts showed the most remarkable activity. The plants can therefore be further subjected to isolation of the therapeutic antimicrobials and pharmacological evaluation be carried out on the purified compounds. The use of the *Vernonia adoensis* by certain communities is justified because of the presence of the phytoconstituents that are active against *Staphylococcus*, a common bacterial causative agent in wounds.

### 5.3.2 Antimicrobial Screening of Crude Endophytic Extracts

In the present study, five crude extracts of endophytic fungal and bacterial species were tested against the human bacterial and fungal pathogens by well diffusion method. All crude extracts in ethyl acetate of the endophytic fungal and bacterial species showed activity against human bacterial and fungal pathogens**.** This study is in agreement with Desale *et al.*, (2013) where fungal endophytes from *Ocimum sanctum* L., screened for the antimicrobial potential of the metabolites produced from the endophytes that showed an antimicrobial activity against *E. coli* followed by *K. pneumoniae* and *S. aureus.*

In this study the most sensitive test microorganisms in the *Ocimum kilimandscharicum* endophytic extracts were the *Candida albicans* and *Klebsiella pneumonia* from two fungal extracts while *Klebsiella pneumonia* and *Escherichia coli* were sensitive in the *Vernonia adoensis* fungal extracts. The bacterial extract in the *Vernonia adoensis* showed antifungal activity against the *Candida albicans*. Two of each of the endophytic fungal extracts from each of the plant species exhibited significant antimicrobial activity. This study helps to justify the traditional use of *Ocimum kilimandscharicum* and *Vernonia adoensis* against human pathogenic bacteria. It has also confirmed that the antimicrobial activity is attributable to the presence of endophytic fungi in the plant. It also leads to the justification that the studies on isolation and

identification of the bioactive compounds can be an important and crucial approach to search for novel natural products.

### 5.4 Minimum Inhibition Concentration (MIC) & Minimum Bactericidal Concentration/Fungicidal Concentration (MBC/MFC) of the plant extracts.

The data obtained through MIC revealed variability in the inhibitory concentrations of each extract for given bacteria and fungi. The MIC values of different plant extracts against the tested bacterial isolates were found in the range of 12.5-25.0 mg/ml for *Staphylococcus aureus* and *Escherichia coli* in chloroform and ethanol extracts respectively for the *Ocimum kilimandscharicum* extracts. In the *Vernonia adoensis* extracts, the MIC was found in the range of 25.0-50.0 mg/ml for *Staphylococcus aureus* and *Escherichia coli* respectively. The most active plant extract against *S. aureus* was the chloroform and ethanol extracts of the *V. adoensis* (MIC value 25 mg/ml) followed by the extracts of *Ocimum Kilimandscharicum* (MIC values 12.5-25 mg/ml). Values for MICs were dependent on the bacterial species. However, ampicillin was a more potent antibacterial than all the extracts with MIC values ranging from 1.25 mg/ml to 5.00 mg/ml in both *Ocimum kilimandscharicum* and *Vernonia adoensis* extracts. None of the extracts from the plants showed any MIC activity against Candida albicans.

From the MIC and MBC assays gram- negative species seemed to be more resistant to plant extracts than the gram positive species as indicated by their MIC and MBC values**.** Results from this study agree with the findings of Stavri *et al.*,(2007) and Doughari (2012) which showed that gram- negative bacteria are less sensitive to antibiotics than gram positive strains. This could easily be attributed to the fact that Gram- negative bacteria possess thick outer membranes that

are highly hydrophobic, providing these organisms with a permeability barrier especially towards hydrophilic compounds such as macrolide compounds including erythromycin (Bennett *et al.,* 2014). Some of the extracts used in this research have been used in other researches as well as in separate ecological zones in the world. Vernonia species possesses antibacterial properties against *Klebsiella pneumonia* and *Mycobacterium tuberculosis*(Kisangau *et al.* 2008; Maregesi *et al.* 2008) and were used in the cure of tuberculosis. A crude extract of *Vernonia adoensis* was found to have high activity against *E.coli*, a gram negative bacterial species of clinical importance (Kisangau *et al*. 2008) which agrees with the findings in this study.

### 5.5 Minimum Inhibition Concentration (MIC) & Minimum Bactericidal Concentration/Fungicidal Concentration (MBC/MFC) of the endophytic extracts.

The lowest concentration of the endophytic extracts at which no growth of microorganism observed upon visual observation after incubation at 37°C for 18 h was considered as the MBC value. The MBC value of the extracts in this study was 50 mg/ml of *Klebsiella pneumoniae* and 25mg/ml for *Candida albicans* in the *Ocimum kilimandscharicum*. In the *Vernonia adoensis*, the MBCs were 25mg/ml and 50mg/ml for *Klebsiella pneumoniae* and *Candida albicans* tested respectively. The lowest MBC value of 25mg/ml was observed in *Candida albicans* for *Ocimum kilimandscharicum* and *Klebsiella pneumoniae* for *Vernonia adoensis* endophytic extracts.

The MBC results showed a significant activity in the extracts from the *Vernonia adoensis* against *Klebsiella pneumoniae* and *Candida albicans* compared to those from the *Ocimum kilimandscharicum.* These MBC results showed that the extracts of the two plants exerted a moderate antimicrobial activity on the strains of *Escherichia coli* and *Staphylococcus aureus* for the crude plant phytochemicals from *Ocimum kilimandscharicum* and *Klebsiella pneumoniae*

and *Candida albicans* from *Vernonia adoensis* of the endophytic extracts at different concentrations**.**

It is worth to note that MBC values obtained for the extracts against the pathogens are higher than MIC, indicating that the extracts are bacteriostatics at lower concentrations and bactericidal at higher concentrations. This suggests that these plant extracts, when used traditionally as antimicrobials inhibit bacterial and fungal growth without necessarily killing them and since most of the traditional preparations lack specific concentrations, this may thus account for the use of large quantity of the extracts by traditional medical practitioners for the treatment of their patients. These observations compare with the findings of the study by Sen *et al.*,(2012), Demain (1999) and Avinash *et al*. (2015). The results obtained in this study indicate that endophytic microorganisms isolated from *Ocimum kilimandscharicum* and *Vernonia adoensis* medicinal plants produced some crude extracts that possess antimicrobial compounds against pathogenic bacteria and fungi. This data supports the general scientific opinion that endophytic microorganisms of medicinal plants are potential sources of bioactive compounds.

# CHAPTER SIX

# SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

## 6.1 Summary of Results

This study shows that both plants had alkaloids, terpenoids, tannins, steroids, saponins and phenols. Chloroform and ethanol extracts showed the highest phytoconstituents with hexane having the least in both plants. Terpenoids and phenols were the most dominant phytoconstituents in the extracts. *Ocimum kilimandscharicum* leaves showed more phytoconstituents than *Vernonia adoensis* leaves and the opposite was observed for the stems. The study also showed that the medicinal plants had culturable endophytes with *Ocimum kilimandscharicum* showing more endophytes than *Vernonia adoensis.* One of the two bacterial endophytes in the *Ocimum kilimandscharicum* died on subculture and was not revived in the subsequent subcultures. The fungal isolates in both plants showed terpenoids, flavonoids and alkalloids with *Ocimum kilimandscharicum* having more endophytes with phytochemicals than *Vernonia adoensis.* Bacterial endophytes showed alkalloids and flavonoids in *Ocimum kilimandscharicum* while alkalloids and terpenoids were found in bacteria from *Vernonia adoensis*. The secondary metabolites from the plants and endophytes showed a significant antimicrobial activity against the tested bacteria and fungi. MIC and MBC results showed that the metabolites are potential antimicrobials against the tested bacteria and fungi.

## 6.2 Conclusions and Recommendations

### 6.21 Conclusions

The current study showed that:

1. Both plants possess active components of medicinal values and provide a partial rationale for the use of these medicinal plants in traditional medicines

2. The isolated endophytes may be a good source of secondary metabolites. Endophytic extracts have great potential as antimicrobial compounds against microorganisms.

3. Both plants can be used in the treatment of infectious diseases and could help to manage microbial infections.

### 6.2.2 Recommendations

The current study recommends that:

1. A detailed bioassay-guided phytochemical study be done in a bid to identify and characterize bioactive secondary metabolites which can be used as templates for new drugs development programmes or as markers for standardization of antimicrobial herbal remedies.
2. More research is needed to isolate and identify the bioactive compounds from the Kakamega forest medicinal plants and associated endophytes that may be used in the agricultural, pharmaceutical and biotechnological research.
3. There is need to carry out an in-depth research about the antimicrobial potency of the purified bioactive compounds from the medicinal plants and endophytes from the plants.

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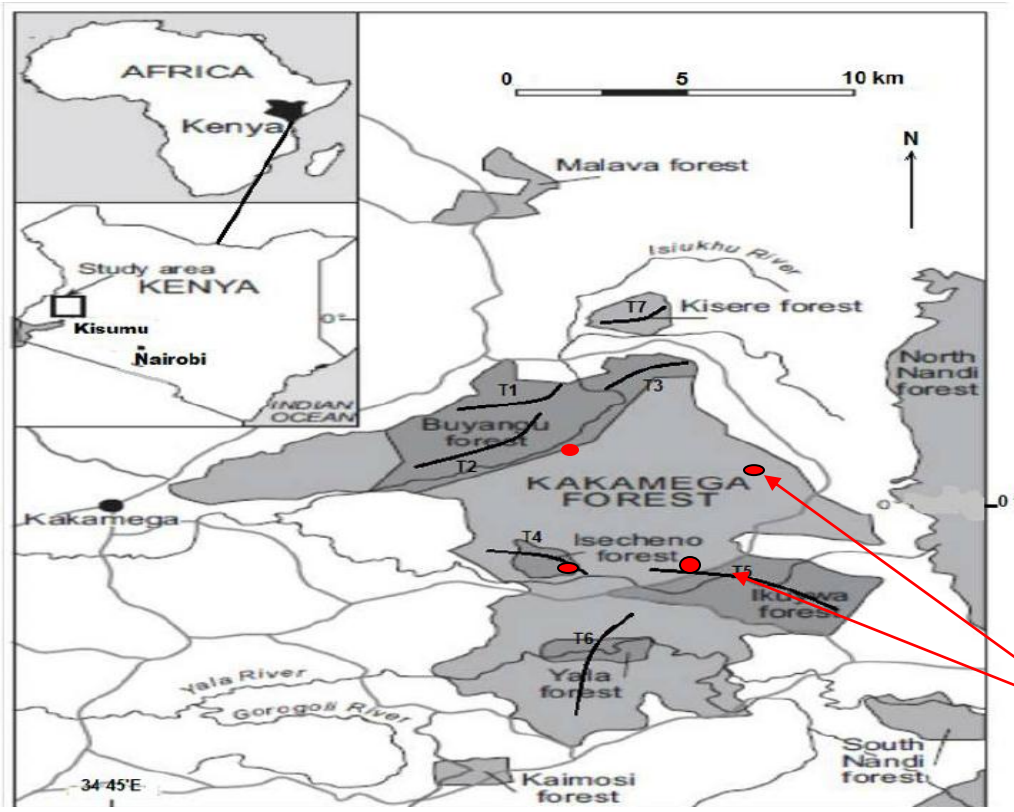
# APPENDICES

## Appendix 1: Medicinal Plants

**Plate 4*: Vernonia adoensis***

**Plate 5*: Ocimum kilimandscharicum***

**Appendix 2: Map of Kakamega Forest Block**




***Map of Kakamega Forest showing the various blocks (Adopted from Munyekenye et al.,* 2008)**

**Appendix 3: Questionnaire**

The purpose of this questionnaire is to know how the traditional herbalists around the Kakamega Forest in Kenya have used the medicinal plants in the management of common diseases among their clients. The information thereafter gathered will help researchers to relate the diseases the medicinal plants treat in the traditional medicine in their pursuit for drug bioprospecting from the natural products of the plants origin.

Therefore this tool has no other intentions other than the above stated purpose.

**Instructions:**

You are expected to circle/tick in the box for your correct answer(s)

1. Gender of the respondent: Male ( ) Female ( )
2. Age in years of the respondent Less than 30 ( ) 30-45 ( ) Above 45 ( )
3. What is your level of education? None ( ) Primary ( ) High School ( ) University ( )
4. Are you a traditional herbalist? Yes ( ) No ( )
5. If yes to Q4, for how long have you been practicing herbalism? Less than 10 years ( ) 10- 20 years ( ) Over 20 years ( )
6. Which herbal plants have you used commonly to your clients? State the number of herbal plants One ( ) Two ( ) Many ( )
7. What are the complaints they present with when they come? Chest complications ( ) STI ( ) Diarrhoea ( ) Others ( )
8. How do you come to know that the herbal plant you are picking is the right one? Indigenous knowledge ( ) Experience ( ) Educational Information ( ) Assisted ( )
9. Where in the forest do you collect the herbal plants? Inside ( ) Periphery ( )

10. What is the age of the herbal plant you collect for your use? Young ( ) Old ( )
11. Which are the parts of the plant that are commonly used? Bark ( ) Stem ( ) Leaves ( ) Flowers ( )
12. How do you obtain the extracts for use to your clients? Soaking in water ( ) Soaking in alcohol ( ) Heating in alcohol ( ) Others ( )
13. Is there a history of your clients using the herbal concoctions and conventional drugs at the same time? Yes ( ) No ( )
14. Are there any cases of your clients reporting back with relapses of the same complications? Yes ( ) No ( )

Thank you for responses

## Appendix 4: NCST Award

REPUBLIC OF KENYA

# NATIONAL COUNCIL FOR SCIENCE AND TECHNOLOGY

Telegrams: "SCIENCETECH", Nairobi
Telephone: 254-020-241349, 2213102
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When replying please quote

P.O. Box 30623-00100
NAIROBI-KENYA
Website: www.ncst.go.ke

Our Ref: NCST/5/003/CALL2/48

Date: 10th May, 2010

Dr. Daniel Namasaka Siamba
Masinde Muliro University of Science & Technology,
P.O. Box 190,
KAKAMEGA

RE: ST&I GRANT

I would like to congratulate you for the award of Science, Technology and Innovation grant for your research proposal.

The Research Committee of the National Council for Science Technology has approved an amount of Kenya shillings **1,500,000/=** towards your proposal titled ***"Isolation, characterization and evaluation of endophytes from selected medicinal plants for anthelmintic metabolites"***.

You are hereby requested to make the necessary adjustments in your proposal and budget in line with the ***reviewers comments*** attached hereto.

Find the enclosed ***Research Grant Contract Form (RIG/03A)*** that should be duly completed and sent back to the National Council for Science & Technology. You should attach a copy of your: - ***National Identity Card, details of the work plan, breakdown of the quarterly budget and an acceptance letter.***

Your acceptance letter and Contract Form should reach us not later than **25th May 2010** for further action.

PROF. SHAUKAT A. ABDULRAZAK, Ph.D, FIBiol, MBS
SECRETARY/CEO

cc. VC
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