EVALUATION OF YIELD, CHEMICAL COMPOSITION AND ANTIMICROBIAL ACTIVITY OF ESSENTIAL OILS OF *Tagetes minuta* L. (ASTERACEAE) AGAINST SELECTED PHYTOPATHOGENS

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DECLARATION

I, the undersigned declare that this research is my original work and has not been previously submitted in this or any other University or Institution for academic credit. All information obtained from other sources has been duly acknowledged.

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DEDICATION

This work is dedicated to my parents Henry and Agnes and my siblings, Mercy and Peter. These were my very first teachers in life and everything that I have learnt is thanks to the good foundation they helped to establish in me.

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LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of variance
CFU	Colony Forming Units
CLSI	Clinical Laboratory and Standards Institute
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EOs	Essential oils
FAO	Food and Agriculture organization of the United Nations
GC	Gas Chromatography
GC/MS	Gas Chromatography- Mass Spectrometry
HPLC	High Performance Liquid Chromatography
ICIPE	International Centre for Insect Physiology and Ecology
IPM	Integrated Pest Management
IUPAC	International Union of Pure and Applied Chemistry
KALRO	Kenya Agricultural and Livestock Research Organization
KI	Kovats retention indices
LSD	Least Significant Difference
MASL	Meters above sea level
MBC	Minimum Bactericidal Concentrations
MHA	Muller Hinton Agar
MFC	Minimum Fungicidal Concentration
MHB	Muller Hinton Broth
MIC	Minimum Inhibitory Concentrations
MS	Mass Spectrometry
MWECAU	Mwenge Catholic University
NA	Nutrient Agar
NCCLS	National Committee for Clinical Laboratory Standards
NIST	National Institute of Standards and Technology
NTP	Normal Temperature and Pressure
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
RNA	Ribonucleic Acid
RSD	Relative Standard Deviation
RT	Retention Time
SG	Specific Gravity
TLC	Thin Layer Chromatography
WFS	World Federation of Scientists
WHO	World Health Organization

ABSTRACT

Phytopathogenic fungi and bacteria cause enormous crop yield losses worldwide. Over the years, management of plant pathogens has primarily relied on the use of synthetic chemical antimicrobials and development of disease resistant varieties. Exploration for biologically active compounds from plants with an aim of discovery and development of novel and eco-friendly biopesticides to combat current and emerging phytopathogens has received increased interest in the recent past. This study aimed at extraction of *Tagetes minuta* essential oils (EOs), evaluation of the antimicrobial activity of the essential oils against selected phytopathogenic fungi and bacteria and characterization of chemical composition of the essential oils. The antimicrobial activity of the essential oils was evaluated against five plant pathogenic fungi: *Fusarium oxysporum, Fusarium solani, Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus niger;* as well as three plant pathogenic bacteria: *Pseudomonas savastanoi* pv. *phaseolicola, Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas axonopodis* pv. *manihotis.* Aerial parts (leaves, flowers and stems) of *Tagetes minuta* plant materials were sampled from four sites within Maseno area, Kisumu County, Kenya. Extraction of EOs was carried out using the steam distillation method in a modified Clevenger-type apparatus. The antimicrobial activity of the oils was assessed by disc diffusion method while gas chromatography - mass spectrometry (GC/MS) was used for characterization of chemical components of EOs. A mean oil yield of 0.0594% w/w was obtained while GC/MS analysis identified 20 compounds corresponding to 96% of the total essential oil. The EOs mainly contained a mixture of monoterpenes (70%) and sesquiterpenes (30%). The most abundant monoterpenes were (E)-Tagetone (11.8%), dihydrotagetone (10.7%), Allo-Ocimene (8.8%), (Z)-β-Ocimene (7.0%) and Limonene (5.3%). Sesquiterpenes concentrations were in the range of 3.4 to 3.5%. Among the identified compounds were Elixene and Silphiperfol-6-ene, two sesquiterpenes being reported for the first time in essential oils of *T. minuta.* The EOs exhibited potent antimicrobial activities against all the studied pathogens. In the case of fungi, the highest activity of the oils was observed in *F.oxysporum* and *A. niger* where mean inhibition zones of 28.67mm were recorded after five days of incubation. The EOs minimum inhibitory concentrations and minimum fungicidal concentrations were in the ranges of 24 - 95mg/ml and 24 - 190mg/ml, respectively. *Pseudomonas savastanoi* pv. *phaseolicola* was the most susceptible bacteria to the EOs, with a mean inhibition zone diameters of 41.83 and 44.83mm after 24 and 48 hours, respectively.

The EOs minimum inhibitory concentrations and minimum bactericidal concentrations were in the ranges of 24 - 48mg/ml and 95 - 190 mg/ml, respectively. These findings provide the scientific basis for the use of *T. minuta* essential oils in the management of economically important plant pathogens. This study thus lays down significant groundwork for a more comprehensive study on the practical feasibility of using *Tagetes minuta* EOs as biopesticide. It is hoped that such studies would potentially promote the development of novel, affordable and eco-friendly biopesticides for management of economically important phytopathogens.

CHAPTER ONE: INTRODUCTION

1.1 Background of the study

Global agricultural losses due to abiotic and biotic factors have been estimated at more than 35% of annual production (Larrañaga *et al*., 2012). In the Unites States alone, pests, plant pathogens and weeds destroy 37% of all potential crops with losses due to insect pests being 13%, plant pathogens 12% and weeds 12%. Among the abiotic factors, pathogenic fungi, bacteria and viruses are the major infectious agents of plants that cause enormous economic losses to farmers worldwide by destroying millions of tons of crop yield annually (Oerke, 2006). Accurate figures of estimated losses due to phytopathogens are hard to obtain and validate in most areas mainly because much of the available data on losses, potential and actual losses (i.e. losses despite the presence of protection practices) are based on combined losses attributable to all pest groups namely weeds, insect pests and pathogens. Moreover, some losses go unnoticed in remote areas. The estimated total global crop losses due to the aforementioned agents vary from about 50% in wheat to more than 80% in cotton with losses due to pathogens alone estimated to be about 16% (Oerke, 2006).

Among all the groups of phytopathogens, fungi are the most important in term of their impact on crop productivity (Mahy and Van Regenmortel, 2010; Fletcher *et al*., 2010). Fungi are a broad group of eukaryotic microorganisms, majority of which are microscopic and filamentous. While it is estimated that there are more than 1.5 million fungal species on earth, only around 15,000 of them cause disease in plants, with the majority belonging to the ascomycetes and basidiomycetes groups (Aiyere, 2004). Fungal pathogens are estimated to be responsible for 40-60% of the total plant pathogenic losses, with losses due to both pre- and post-harvest fungal diseases estimated to be more than 200 billion Euros annually in the United State (Bau *et al*., 2003).

The threat of plant pathogenic bacteria to food security is to some extent less in comparison to that posed by fungi (Agrios, 2005). In terms of the number of species, fewer bacterial species cause plant diseases than fungal ones. The estimated number of phytopathogenic bacteria is about 100 species (Agrios, 2005). Nevertheless, the economic impact of phytopathogenic bacteria cannot be underestimated and a single bacterial species can be responsible for hundreds of different plant diseases. For example, about 350 different plant diseases are caused by the species of *Xanthomonas* (Leyns *et al.,* 1984).

The modern agricultural system of crop protection and management against phytopathogens has primarily over relied on the uses of synthetic chemical pesticides (Larrañaga *et al*., 2012). The use of synthetic pesticides has greatly boosted crop yields and thus increased global food security. However, it has over the years become apparent that over reliance on these chemicals in agriculture is not without serious environmental and health concerns (Horrigan *et al*., 2002). There is thus a need to focus more on sustainable and cost-effective methods of reducing plant losses occasioned by phytopathogens both during the production and post-harvest stages. One way of doing this is through research geared towards the development of novel plant-derived biopesticides that are both environmentally biocompatible and effective against current and emerging phytopathogens.

1.2 Problem statement

The world population is increasing rapidly and with it, the challenge of ensuring food security to all is both acute and chronic depending on the region. The question of having a food-secure world that produces enough food for everyone and thus ensures that the ever increasing global food consumption demands are met is recognized as one of the existential challenges for the sustainable development of humanity today (Bouma and McBratney, 2013). The worldwide, per capita availability of food is estimated to increase by about 7% by the year 2050. During this period, per capita availability of food for sub-Saharan Africa is projected to be about 2708 kcal, which will be just above the bare minimum recommended requirement (FAO, 2011). There is thus a need to increase crop productivity in order

to fulfill the dietary needs of majority of the people especially in the developing countries where the population is fast growing. This is especially pertinent today given the complex array of emerging issue that have a direct impact on crop productivity, chief among them the threat posed by plant pathogens.

Plants are always exposed to diverse groups of microorganisms. Some such as mycorrhizae and nitrogen fixing bacteria form beneficial associations with plants enabling them to obtain essential nutrients such as phosphorus and nitrogen. Indeed, some plant beneficial microbes such as rhizobacteria and mycorrhizal fungi have been shown to help plants resist the attacks by pathogens and herbivorous insect in addition to enabling them tolerate abiotic stress (Pineda *et al*., 2013). Many other plant-associated microorganisms however, are pathogens responsible for diseases that continue to be a major challenge to global agricultural production resulting in huge economic losses.

The quest for new antimicrobial agents to combat current and emerging phytopathogens is an ongoing endeavor among scientists. Exploration for biologically active compounds from plants aimed at discovery and development of novel antimicrobial agents is one area that has in the recent past received increased interest. Many research reports have highlighted the potential of botanicals as future sources for the development of eco-friendly biopesticides for crop protection (González-Lamothe *et al*., 2009; Raja, 2014). This study was aimed at the extraction, antimicrobial assessment of *Tagetes minuta* essential oils against some phytopathogenic fungi and bacteria and chemical characterization of the essential oils.

1.3 Justification

To reduce crop yield losses occasioned by pathogens and diseases, modern agricultural system depends to a greater extent on the use of synthetic chemical pesticides in addition to development of disease-resistant varieties. Despite these efforts however, phytopathogenic fungi and bacteria contribute significantly to crop yield losses through direct crop destruction and/or capital spent on chemical inputs

(González-Lamothe *et al*., 2009). In the United States alone for example, over $600 million is spent annually on fungicides (Arora *et al*., 2004). Moreover, the benefits of using synthetic pesticides are mostly based on direct crop returns and are thus overestimated because they do not include indirect environmental, health and economic cost associated with their application (Pimentel, 2005). Contamination of underground water, rivers, lakes, air and soil by pesticides is a major environmental concern today. Many chemical pesticides have additionally been found to be toxic to non-target organisms such as birds, beneficial insects such as bees and non-target plants (Aktar *et al*., 2009). With regard to human health, clinical and epidemiological studies have established a cause-effect relationship between pesticides and a wide range of human diseases especially in dermatological, neurological, behavioural, reproductive and developmental systems (Gupta, 2008). Additionally, certain synthetic chemical pesticides have been found to be carcinogenic to humans and wild animals (Daoubi *et al*., 2005). These among other negative side effects associated with chemical synthetic pesticides have led to an increase in the need for exploration of alternative options that are generally cheaper, readily available, sustainable, and that pose the least negative effects to human and livestock health and to the environment.

A number of factors have contributed to increase in interest in plants as potential source of control agents for phytopathogens. To begin with, plant-derived antimicrobials have been found to be more adaptable and safer since they have low mammalian toxicity (Amini *et al.,* 2012). Moreover, botanical fungicides and bactericides are easily degradable and hence environmental-friendly and have a high potential for use in integrated pest management (IPM) programs, resulting in their wide public acceptance (Katooli *et al*., 2011; Al-Samarrai *et al*., 2012). Synthetic pesticides on the other hand are associated with numerous drawbacks such as; development of resistance among target pathogens as a result of overuse. Most synthetic pesticides have longer environmental retention and thus are more likely to kill beneficial organisms. Furthermore, most synthetic fungicides and bactericides are liable to remain in plant tissues and products such as fruits and vegetables long after the application posing a great health risk to human, animals and the

environment in general (Al-Samarrai *et al*., 2012). Furthermore, synthetic agricultural chemicals are generally overpriced hence reducing their availability to small scale farmers especially in the developing countries (Abdallah, 2011; Katooli *et al*., 2011).

There is therefore a continuous need to evaluate plants and plant secondary metabolites such as essential oils (EOs) from promising plants and indeed other natural products as potential agrochemical fungicides and bactericides and as alternatives to synthetic antimicrobial agents. Already, some studies on *Tagetes minuta* essential oils as potential biopesticide have shown promising activity against economically important phytopathogenic fungi such as *Pyricularia grisea, Sclerotium rolfsii, Alternaria solani, Aspergillus niger* and *Fusarium oxysporum* (Saha *et al*., 2012; Shirazi *et al*., 2014).

1.4 Objectives

1.4.1 Broad objective

To determine the percentage yield and identity of the chemical composition of *Tagetes minuta* essential oils and to evaluate the antimicrobial activity of the essential oils against selected phytopathogenic fungi and bacteria.

1.4.2 Specific objectives

i. To determine the percentage yields of crude essential oils extracted from leaves, flowers and stems of *Tagetes minuta.*

ii. To assess the antimicrobial effect of crude essential oils of *Tagetes minuta* against selected phytopathogenic fungi and bacteria.

iii. To characterize the chemical composition of the essential oils of *Tagetes minuta*.

1.5 Hypothesis

Tagetes minuta contains essential oils with bioactivity against economically important phytopathogenic fungi and bacteria.

CHAPTER TWO: LITERATURE REVIEW

2.1 Major plant pathogens

In different historical periods, plant pathogens have had a profound effect on civilization through destruction of crops resulting in starvation, mass migration and massive loss of human lives. Many famines of historical significance have often been a result of the destruction of food crops by either environment extremes or through damage caused by pests, pathogens and diseases (Brown and Ogle, 1997). A good example is the devastating Irish potato famine of the 19^{th}century. This epidemic which was caused by destruction of potato by the late blight disease resulted in the death of over a million people and a loss of nearly a third of Ireland's population (Clement, 1995). The causal organism was later identified as *Phytophthora infestans* an oomycete, which till today remains one of the most important pathogens of potato and tomato. Since then, several major plant disease epidemics have occurred in different parts of the globe sometimes resulting in total collapse of local and regional industries that depend on the affected plants. Moreover, plant disease epidemics have at times forced people in the affected areas to completely alter their forms of livelihood by making it untenable to grow the susceptible crops without the use expensive and at times environmentally-destructive methods (Brown and Ogle, 1997).

Plant pathogens belong to different groups and taxa. The major plant pathogenic organisms are broadly classified under fungi, oomycetes, bacteria, viruses, mycoplasma, nematode, protozoa and even parasitic plants (Rangaswami and Mahadevan, 1999). Although the most widely studied plant pathogens are those that attack and cause diseases in food crops, pathogens attack nearly all plants from ornamental plants to plants in natural ecosystems (Fletcher *et al.,* 2010). The relationships and interactions that result in a plant diseases as a result of pathogen attack are often complex. However, three interactive components must be present in order for a particular plant disease to occur; the pathogen itself, a susceptible plant host and a favorable environment permitting infection of the host. The interaction between the above three factors have been conceptualized into the disease triangle,

an important empirical tool that can be used to understand the dynamics of disease-causing plant pathogens, predict epidemiological outcomes in plant health and perform other complex analysis of the relationships between the environment, the host and the pathogen (Scholthof, 2007).

Most plant pathogens display enormous diversity in their life-history strategies, host range and host-pathogen interactions (Burdon and Silk, 1997). In terms of host range, certain phytopathogens have a narrow host range e.g. restricted to a single plant species. Others may infect a single plant genus while there are those that can attack a large number of hosts that can span many plant genera and families (Clement, 1995). In terms of host-pathogen relationships and interactions, plant pathogenic microorganisms can be divided into either obligate parasites (biotrophs) or non-obligate parasites (hemibiotrophs and necrotrophs) (Agrios 1997). Biotrophs are obligatory pathogens that require plant tissue that is actively metabolizing in order to survive. All of the more than 700 known plant viruses are biotrophs. Many of these infectious particles contain ribonucleic acid (RNA) rather than deoxyribonucleic acid (DNA) and are obligatory pathogens that cannot live or replicated outside susceptible plant host (Fletcher *et al.*, 2010). Moreover, most plant viruses are transmitted by insect vectors and for this reason, their epidemiology is mostly dependent on the range, behavior and general biology of the transmitting insect vectors (Fletcher *et al.,* 2010). In addition to viruses, some fungi (e.g. rust, smut, powdery and mildew fungi) and bacteria (e.g. hairy root disease-inducing *Agrobacterium rhizogenes*) are biotrophs. Hemibiotrophs pathogens (e.g. *Monilia fructigena)* on the other hand, are dependent on their living host until necessary growth and reproduction has occurred (Lehtonen, 2009).

2.1.1 Plant pathogenic fungi

Among all plant pathogens, fungi are responsible for the greatest destruction of plants both in agricultural and natural ecosystems (Fletcher *et al.*, 2010). Being heterotrophic, fungi lack chlorophyll and reproduce by sexual or asexual spores. Many members of a large group of fungi called deuteromycetes however, do not produce spores or the sexual phase is rare or still unknown (Agrios 1997). Many

plant pathogenic fungi are capable of infecting any plant tissue during any stage of growth. Most have complex life/infection cycles involving multiple (up to five) phases each of which may occurs on a different plant host and may be characterized by different reproductive strategies (Fletcher *et al*., 2010). The presence of both sexual and asexual reproductive stages allow most phytopathogenic fungi to infect plants in different climatic zones from the hot and dry arid zones to the wet zones in the tropic and equatorial regions (Agrios, 2005).

2.1.2 Plant pathogenic bacteria

Plant pathogenic bacteria are mainly heterotrophic microorganisms, which develop on/in host plants as parasites (Sobiczewski, 2008). Phytopathogenic bacteria may belong to either the Eubacteria group whose members possess a cell wall and can be cultured in artificial media or mollicutes, also known as mycoplasma, a class of wall-less bacteria surrounded by a cell membrane only (Sobiczewski, 2008). Most plant pathogenic bacteria including members of the genera *Pseudomonas* and *Xanthomonas* have a life cycle that include an epiphytic phases during which they form colonies on plant surfaces. Members having such a life cycle will only enter the plant interior once conditions become favourable (Fletcher *et al*., 2010). The infection process typically occurs through various natural opening such as stomata, lenticels, hydathodes, and nectaroides in addition to injures or wounds (Chaube and Pundhir, 2005). Other bacterial pathogens such as the wall-less phytoplasmas are usually introduced directly into the host plant tissues mostly via plant-feeding insect vectors (Sobiczewski, 2008).

The most important bacterial plant pathogens are small rod-shaped (*Streptomyces* which are filamentous are an exception) about 0.7-3.0 x 0.4-1.5 µm (Brown and Ogle, 1997). The most significant Gram-negative genera of bacterial plant pathogens are *Agrobacterium, Erwinia, Pseudomonas* and *Xanthomonas.* All the four genera are single-celled, non-spore forming rods with the size of approximately 2.0 x 0.7 µm. Gram-positive phytopathogenic bacteria on the other hand are mostly represented by members of four genera namely*; Arthrobacter, Clavibacter, Curtobacterium* and *Rhodococcus* all of which are aerobic (Brown and Ogle, 1997).

2.2 Plants as sources of antimicrobial agents

Microorganisms such as bacteria, fungi, protozoa and viruses have existed on earth for million years and are in fact considered the oldest and most adaptive creature in nature (Abdallah, 2011). The long history of the existence of microorganisms is closely tied with that of the struggle by mankind to control and/or contain those microbes that are pathogenic to human, livestock and crops. Fossil records have revealed that human beings living in Mesopotamia more than 60 000 years ago were using medicinal plants to treat various infections (Cowan, 1999). This is an indication that faced with the challenge of microbial infections, perhaps plants and plant products were the first curative and preventive defense weapons that mankind turned to (Abdallah, 2011). The search for, and development of new antimicrobial agents to fight current and emerging phytopathogens has thus continued throughout much of the recent history. Currently, in additional to synthetic and other natural sources, plants serves as a starting point for the discovery and development of many antimicrobial formulations some of which have been successfully tested and commercialized. An example is azadirachtin, a tetranortri-terpenoid limonoid which is extracted from the seeds of neem (*Azadirachta indica* A. Juss). Azadirachtin has insecticidal and anti-feedant effects in several insects and is a constituent of some plant-derived pesticides (Raja, 2014). Among the major groups of plant compounds that have elicited a lot of interests among researchers are secondary metabolites such as essential oils (Bassolé and Juliani 2012; Raja, 2014).

2.3 Plant essential oils

Plant essential oils (EOs) also known as volatile oils or ethereal oils are volatile, natural aromatic complexes, formed by certain plants as secondary metabolites (Prabuseenivasan *et al*., 2006). Essential oils are isolated from various parts of the plant, such as leaves (basil, patchouli, cedar), fruits (citrus), bark (cinnamon), root (ginger), grass (citronella), gum (myrrh and balsam oils), berries (pimenta), fruits (bergamot, orange, lemon, juniper), seed (caraway), flowers (rose and jasmine), twigs (clove stem), wood (amyris), heartwood (cedar), rhizomes (ginger, calamus, curcuma) and saw dust (cedar oil) (Burt, 2004; Hussain *et al*., 2008; Chi, 2013). They are not produced by all plants but rather, their occurrence is restricted to well

over 2000 plant varieties from about 60 different families. However, only about 100 varieties are the basis for the economically important production of essential oils in the world (Chi, 2013).

Essential oils have been studied extensively for their antimicrobial properties among other biological activities. Essential oils containing mixtures of volatile substances, such as monoterpenes, sesquiterpenes and/or phenylpropanoids, esters, alcohols, and terpenoids among other constituents have been reported to have antibacterial, antifungal, antiviral, nematicidal and insecticidal properties (Centeno *et al*., 2010; Silva *et al.,* 2012; Pooja *et al.,* 2013). Since essential oils could contain up to 100 different phytochemicals, they have multiple modes of action against bacteria (Lambert *et al*., 2001). Their antibacterial modes of action includes; interference and destabilization of the phospholipids bilayer of the cell membrane, enzyme systems, and genetic material (Kim *et al*., 1995). Fungal growth inhibition by essential oils just like in the case of bacteria involves multiple modes of action such as prevention/reduction of hyphal growth and sporulation, alteration of cell wall composition, induction of lysis and cytoplasmic evacuation (Kishore *et al.,* 2007).

2.4 Extraction of plant essential oils

Essential oils are extracted from oil sacs in flowers, leaves, stems, roots, seeds, wood, bark and other plant organs. There are numerous methods available for the extraction of EOs such as steam distillation, hydrodistillation, maceration, solvent extraction and cold pressing also known as expression among other methods (Schmidt, 2010). The choice of a particular method will depend on a number of factors such the plant material as well as the desired end-product (Kabura, 2009). Furthermore, the isolation of an essential oil will be facilitated by its various properties as vapor pressure, solubility and polarity among other chemical and physical properties (Okoh, 2010).

Steam distillation is undoubtedly one of the most popular methods of essential oils extraction (Schmidt, 2010). Steam distillation as a technique involves distillation of two immiscible liquids, for which steam provides one of the immiscible phases. The two substances i.e. water and essential oils mix in the gaseous phase and co-distill,

but when cooled the two components separates since they are immiscible (Pavia *et al*., 2005). In this method of essential oil extraction, the plant material is packaged into a still where pressurized steam from boiling water passes through. The heated steam causes globules of oils in the plant to burst and the oils to evaporate. The essential oils and steam vapours passes through the top of the still into a condenser where they are condensed back to liquids with the oils separating from the water and floating on the top (Raaman, 2006). The fundamental nature of this technique is that it enables a compound or mixture of compounds to be distilled at temperature considerably below that of the boiling point(s) of individual constituent(s) (Kumar, 2010). Essential oils typically consist of individual components with boiling points of 200°C and above. However, in the presence of pressurized steam, these constituents are volatilized at temperature close to 100°C, at atmospheric pressure (Rao and Pandey, 2007).

2.5 Methods of characterization of plant essential oils

Different analytical methods have been developed over time for analysis of essential oils with the aim of understanding some other their physical and chemical properties. Most classical analytical methods of essential oils focused mostly on two main properties; identity and purity (Zellner *et al*., 2010). A number of techniques are used to assess the physical properties of essential oils which include; the specific gravity (SG), defined as the ratio of the densities of the oil and that of water at similar temperatures. Other important physical properties of EOs include; optical rotation, refractive index, solubility in various solvent, and melting and boiling points (Zellner *et al*., 2010). Classical techniques have also been used to assess some chemical properties such as the presence of various chemical groups such as halogenated hydrocarbons, heavy metals, aldehydes, ketones and esters (Zellner *et al*., 2010).

Currently, chromatography methods are the most widely used techniques for analysis of plant essential oils. Various chromatographic techniques such high performance liquid chromatography (HPLC), thin layer chromatography (TLC) and gas chromatograph (GC) are used for analysis of essential oils (Zellner *et al*., 2010). Most often however, additional confirmatory procedures are required for reliable identification and characterization of components within a given essential oil (Soran *et al*., 2009). The most widely used method

for the chemical analysis of essential oils is gas chromatography (GC) (qualitative analysis) and gas chromatography-mass spectrometry (GC/MS) (quantitative analysis). The coupling of gas chromatography with a mass spectrometry results in one of the most widely and well-established of the so called hyphenated analytical techniques for essential oils (Kubeczka, 2012). This combination provides a powerful tool that facilitates direct and effectively continuous correlation of chromatographic and mass spectroscopic properties of essential oil components (Zellner *et al*., 2010). The potential for combining gas chromatography and mass spectrometry has highly increased the analytical sensitivity, data acquisition and processing in essential oils characterization. This method of essential oil analysis is very discriminatory because peak identification is further validated thus increasing the precision and accuracy of quantitative essential oils analyses. Moreover, the use of automated data acquisition and processing systems and library search techniques ensures that the enormous amount of data generated by combining the two techniques is fully exploited (Zellner *et al*., 2010). Identification of the main components in an essential oil involves the comparison of both the GC retention times and the MS data against those of the reference mass spectral library, use of retention indices such as the Kovats retention indices (KI) and comparison with previous literature.

2.6 Description of *Tagetes minuta*

2.6.1 Taxonomy and botany of *Tagetes minuta*

The genus *Tagetes* belongs to Asteraceae or Compositae family which comprise of about 28 genera and about 216 species (Barkley *et al*., 2006). All members of genus *Tagetes* are flowering herbaceous plant. The genus is represented by 56 species 27 of which are annual and 29 perennial (Soule, 1993a; Soule, 1993b). Members of the genus have however shown paraphyletic based on molecular phylogeny (Loockerman *et al*., 2003). *Tagetes minuta* L. is known by numerous common names such as Mexican marigold, stinkweed, stinking roger and khaki bush and locally as Ol’ bangi (Maa) Omotiokia (Kisii), Muvangi/Kavangi (Kamba) Nyanjaga (Luo), Mũbangi (Kikuyu), Etakanyi (Luhya) and Chemiasoriet (Kalenjin). *Tagetes minuta* is a strongly scented annual herb reaching heights of 50-150 cm.

The taxonomy of the plant can be summarized as follows (USDA-NRCS, 2016):

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsidia

Order: Asterales

Family: Astraceae

Genus: *Tagetes*

Species: *Tagetes minuta* L.

2.6.2 Economic importance of *Tagetes minuta*

Tagetes minuta plant and its products have had a long history of human usage in beverages, confectionery, raw material for perfume and flavor production, frozen dairy desserts and as puddings and condiments (Soule, 1993a; Soule, 1993b; Wanzala and Ogoma, 2013). Furthermore, *T. minuta* usage is widely reported in the traditional medicinal practices of many communities where various parts of the plant are used in preparations of decoctions and concoctions for treatment of various human and livestock ailments (Makang'a, 2012). A paste made from the plant leaves is for example used for wound healing, as an anti-inflammatory and bronchodilatory (Abbasi *et al.*, 2010). Among the Kamba and Luo communities of Kenya, an infusion made from the plant is used as an antivenin (Owuor and Kisangau, 2006). The Luo additionally uses *T. minuta* in treatment of wounds where whole plant is pounded and applied in wounds (Geissler *et al.,* 2002).

Tagetes minuta has also been used in the control of various crop pests and livestock parasites. In Brazil for example, local farmers are known to intercrop the plant with other crops with the scent of its flowers believe to act as a repellent against a broad range of plant pests such as aphids, caterpillar, mites and moths (Guimarães, and Mourão, 2006). In Uganda similarly, *T. minuta* is recognized by some local community as a pesticidal plant where it is used to control insect pests and nematodes (Mwine *et al.*, 2010). *Tagetes minuta* has also been used customarily by the Bukusu of Western Kenya in control and management of ticks (Wanzala *et al.*, 2014). The plant is additionally used as an ornamental for beautification and landscaping (Sadia *et al.*, 2013). *Tagetes minuta* is also an important agricultural weed of many crops. In part of East Africa for example, the plant has been reportedly found in 10% of

the maize fields. As a weed, *T. minuta* is very aggressive and can totally overwhelm short-statured crops if not controlled early enough. The plant has also been cited as a contaminant in harvested grains such as wheats (Holm *et al*., 1997).

2.6.3 Origin and distribution of *Tagetes minuta*

Tagetes minuta L., is native to the temperate grasslands and montane regions of Southern South America in countries such as Argentina, Chile, Bolivia, Peru, and in the Chaco region of Paraguay (Soule, 1993a). During the Spanish colonization of South America, *T. minuta* was introduced and naturalized around the world in Africa, Asia, Australia, New Zealand, and United States including Hawaii, and islands of Cape Verde, Madeira and Madagascar (Soule, 1993a; Naqinzhad and Mehrvarz, 2007). In the temperate region, *T. minuta* grows naturally from spring and then disappears at the beginning of winter having completed it life cycle (Chamorro *et al*., 2008). *Tagetes minuta* grows easily in disturbed areas during early successional stages and because of its affinity for disturbed areas; it has colonized many areas around the world (Soule, 1993a). Having been introduced in many regions of the world, the plant currently grows under a broad range of climatic conditions from the extreme temperate to tropical regions (Shahzadi, 2012). Indeed, *T. minuta* has become a major agricultural weed in many countries (Sadia *et al*., 2013). It is a problematic weed for pasture and many crops such as maize and wheat within East and South Africa, South America and Australia (Makang'a, 2012).

Due to its robust habit and aggressive competition for space and light, *T. minuta* is resistant to many natural enemies and competitors and once established, it creates a dense monotypic stands displacing other plants around it (Hulina, 2008). The plant generally thrives on light (sandy), medium (loamy) and heavy (clay) soils which are well drained, acidic, neutral and alkaline but can also grow well in shades and in almost every type of soil owing to its competitive nature (Shahzadi, 2012; Makang'a, 2012). In addition to growing wildly *T. minuta* is also cultivated commercially in some countries (Wanzala and Ogoma, 2013). For example, it is grown and harvest for its essential oils in France and North America and Brazil, as a vegetable in Peru, and for the perfumery industry in South Africa (Soule, 1993a; Meshkatalsadat *et al*., 2010). In Kenya, *T. minuta* was first recorded as an alien weed during the 1920s and was primarily restricted to the higher altitudes regions of the country. Today

however, the plant is found in most parts of the country having spread as a result of increasing agricultural activities (Stadler *et al*., 1998).

2.6.4 Phytochemistry of *Tagetes minuta*

A variety of chemical constituents have been isolated from *T. minuta* and their chemical structures elucidated. Most of the isolated compounds belong to the classes as essential oils, flavonoids, saponins, tannins, carotenoids and phenolic compounds (Sadia *et al*., 2015). Essential oils are some of the most widely studies phytochemicals from *T. minuta* largely because of the numerous biological activities attributed to them. Among the main chemical component identified in *Tagetes minuta* essential oils include; dihydrotagetone, ocimenes, ocimenones tagetone and limonene among others (Shahzadi, 2012; Wanzala and Ogoma, 2013; Shirazi *et al*., 2014). Furthermore, the chemical composition of essential oils of *T. minuta* from different regions have been studied and found to vary considerably (Chalchat *et al*., 1995; Senatore *et al*., 2004).

Most studies have reported significance differences in the chemical profiles of essential oils of *T. minuta* based on a number of factors namely; the harvesting location/region (Senatore *et al*., 2004; Chamorro *et al*., 2008), stage of harvest (Chalchat *et al.,* 1995), plant parts used (Chalchat *et al.,* 1995; Chamorro *et al*., 2008) and the climatic conditions under which the plant grows (Mohamed *et al*., 2002). These compositional variations of essential oils from the same plant species results in chemotypes; which are the result of biological variations caused by the effects of different soils, temperature, weather conditions, and light among other factors. This implies that it is possible for the chemical composition of plants that are botanically identical to vary considerably.

2.6.5 Bioactive properties of *Tagetes minuta*

Tagetes minuta and some of its extracts have been studied extensively and a number of them have been credited with having a wide range of bioactive properties such as antimicrobial, insecticidal, nematicidal, acaricidal and allelopathic among other biological activities (Shahzadi *et al*., 2010; Saha *et al*., 2012). *Tagetes minuta* root secretions have been reported for instance to have effective biocidal activity against some subsurface and surface soil pathogens (Hulina, 2008). Essential oils extracted from different parts of the plant in varied

stages of the plants' growth have been found to have biocidal activity against *Varroa destructor*, an ectoparasitic mite of the European honey bee (*Apis mellífera*) (Chamorro *et al*., 2011). A study on the antinematicidal activity of two pure compounds (Z-β-ocimene and dihydrotagetone) isolated from *T. minuta* essential oils against *Meloidogyne incognita*, a plant-parasitic nematode revealed high levels of egg-hatch inhibition and juvenile mortality (Adekunle *et al.,* 2007).

Though little information exist in literature on the activity of *T. minuta* essential oils against plant pathogenic bacteria, some promising antibacterial activity of both aqueous and organic extracts have been reported against a wide range of human pathogenic bacteria such as *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella typhi*, and *Staphylococcus aureus* (Senatore *et al*., 2004; Tahir and Khan, 2012). In the case of fungi, aqueous and organic extracts of *T. minuta* have been reported to have considerable activity against mycotoxigenic fungi such as *Fusarium verticillioides*, *Fusarium proliferatum*, *Aspergillus flavus* and *Aspergillus parasiticus* (Thembo *et al*., 2010). Antifungal activity of the oils have also been reported against phytopathogenic fungi namely such as *Pyricularia grisea, Sclerotium rolfsii, Alternaria solani* and *Fusarium oxysporum* (Saha *et al*., 2012).

CHAPTER THREE: MATERIALS AND METHODS

3.1 Project procedure

The study was designed to start with the collection of plant materials and extraction of the essential oils of *Tagetes minuta*, evaluation of the antimicrobial activity of the oils against selected phytopathogenic fungi and bacteria and characterization of chemical composition of the essential oils in that order. Extraction of essential oils was carried out using the steam distillation method in a modified Clevenger-type apparatus while the antimicrobial activity of the oils was assessed by disc diffusion method. The essential oils were analyzed by gas chromatography-mass spectrometry (GC-MS) for characterization of chemical composition. The project procedure is summarized in the following log frame (Figure 1).

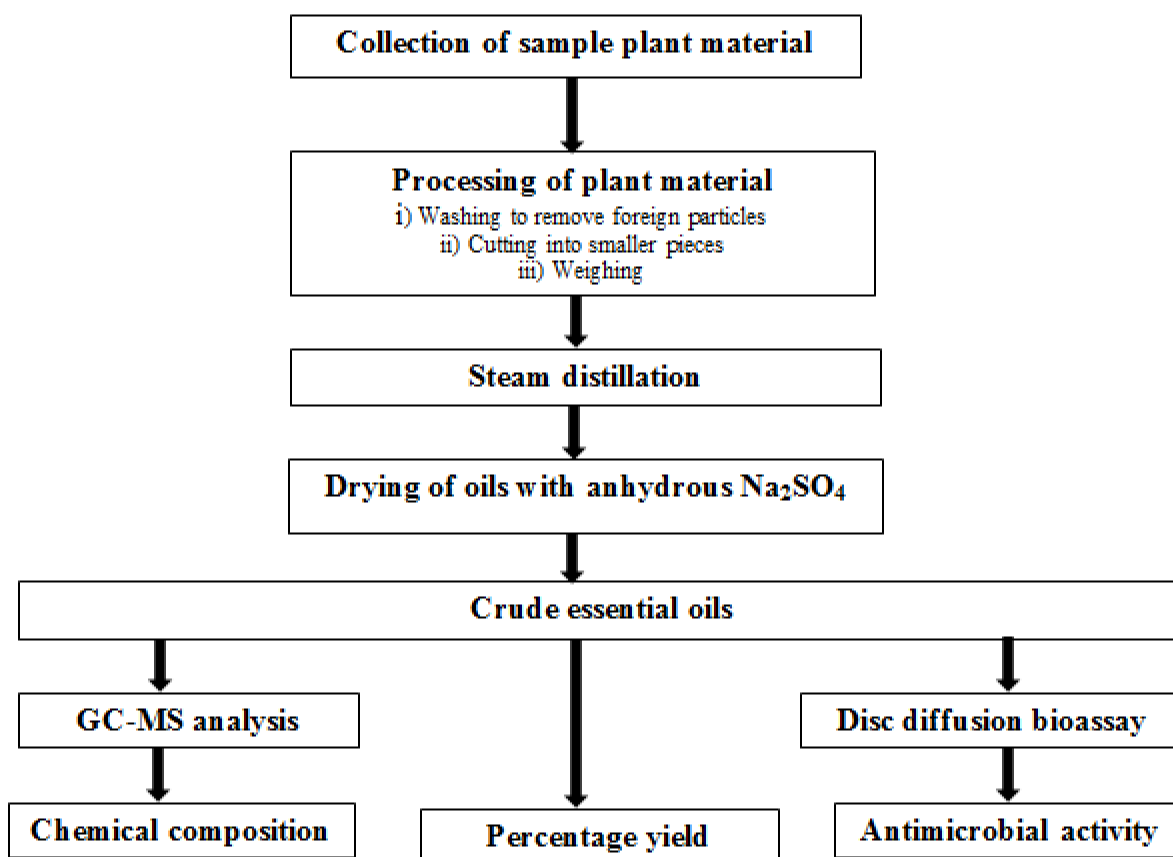

Figure 1: A summary of the study outline showing the research activities and experimental methods

3.2 Collection and authentication of *Tagetes minuta* samples

The aerial parts (leaves, flowers and stems) of *Tagetes minuta* were sampled from four sites within Maseno area ($0^{o}0'21.43''$S, $34^{o}36'6.23''$E, 1524 MASL), Kisumu County, Kenya between October-November, 2015. A minimum of forty plants were sampled at flowering stage from each of the four identified sites. The sampled plant materials were then packaged and transported in Kraft bags from the field to the Chemistry laboratory, Maseno University. The plant materials were washed under running water to remove any foreign materials and then dried on laboratory benches for a few hours in a well ventilated room before extraction of the essential oils. A sample of the collected plant materials was prepared, packaged and stored according to the herbarium rules and regulations. This sample was later taken to the herbarium at the School of Biological Sciences, University of Nairobi, Kenya for identification, authentication and further taxonomic studies. Authentication of the collected plant materials was performed by Mr. Mutiso, a Plant Taxonomist at the School of Biological Sciences, University of Nairobi and a voucher Specimen (MMG2015/01) deposited at the University's herbarium.

3.3 Extraction of essential oils

Extraction of *Tagetes minuta* essential oils was carried out at the Department of Chemistry, Maseno University. Eight liters of water was poured into a 50-liter flat-bottom distillation tank (Figure 2) that is part of the modified Clevenger-type apparatus (Clevenger, 1928). *Tagetes minuta* leaves, flowers and stems were cut into small pieces (≈10cm long), weighed and 4 kg loaded into the still of the tank. The rid of the distillation tank was tightly secured and the plant material subjected to steam distillation. The collection of oil started after a heating time of about 40 minutes and continued until no more essential oil was obtained (5-8 hours). After the distillation process was complete, the volatile essential oils were removed from the top of the hydrosol and dried over anhydrous sodium sulphate (Na_2SO_4) (Golfakhrabadi *et al.*, 2015). The oils were then filtered using Whatman filter paper (No. 1) and collected into 3ml airtight glass vials. The essential oils were transported in a cooler box from Maseno University and stored at -20°C in a deep freezer in the Microbiology laboratory at the School of Biological Sciences, University of Nairobi, until when they were required for chemical analysis and bioassays.

To evaluate the precision of the steam distillation process and thus the efficiency of the extraction apparatus used, both the standard deviation and the relative standard deviation (RSD) were calculated. The % of RSD was used as an indicator of the precision of the steam distillation process: Specification: precision = maximum of 2% (Caburian and Osi, 2010). To determine the density of the essential oil, the weight of 1ml of the essential oil was taken and the value obtained was used to calculate the density of the oil using the formula:

$$\text{Density} = \frac{\text{Mass}}{\text{Volume}}$$

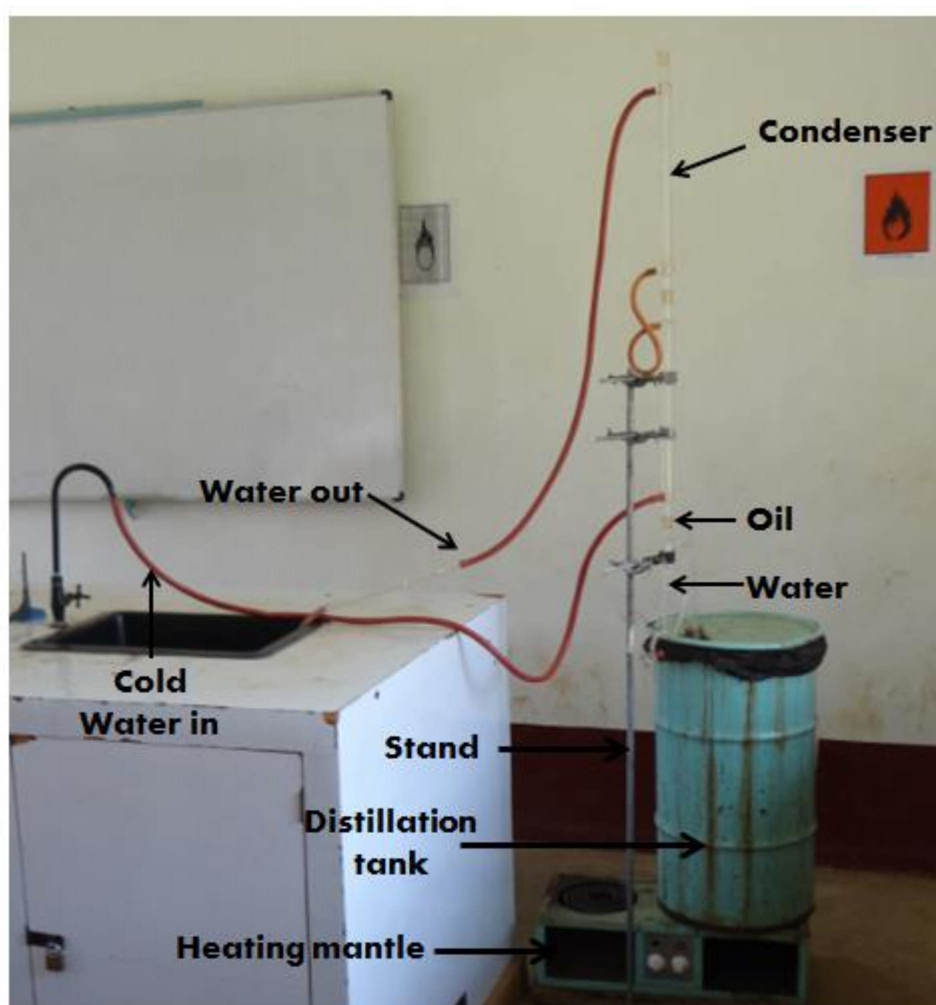

Figure 2: A modified Clevenger-type apparatus that was used to extract the essential oils of *Tagetes minuta*

3.4 Determination of organoleptic properties and solubility of the essential oils

The organoleptic properties of the oils were determined as follows: The essential oils in a transparent glass vial were observed against a white background to

determine the colour and clarity; the characteristic odour/smell was determined by sniffing while, to determine the characteristic feel to the touch, the oil was rubbed between the fingers (Caburian and Osi, 2010). The solubility of the oil in three common solvents namely; ethanol (CH_3CH_2OH), dimethyl sulfoxide (DMSO) and dichloromethane (CH_2Cl_2) was also assessed at the level of 1:1(v/v). Dimethyl sulfoxide was used as a diluent for the essential oils and as a negative control in the antimicrobial activity bioassays while dichloromethane was used in preparation of the stock solution for GC-MS analyses.

3.5 Fungal and bacterial test pathogens

Five economically important fungal plant pathogens - *Fusarium oxysporum, Fusarium solani, Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus niger* - and three bacterial plant pathogens - *Pseudomonas savastanoi* pv. *phaseolicola, Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas axonopodis* pv. *manihotis* were used as test pathogens. The test pathogens were retrieved from the culture collection center at the Plant Pathology Laboratory, Department of Plant Science and Crop Protection, University of Nairobi. Confirmation of the identity of the pathogens was done based on their cultural and morphological characteristics (Klich, 2002; Leslie and Summerell, 2006; Watanabe, 2010).

3.6 Determination of the antimicrobial activity of *Tagetes minuta* essential oil

3.6.1 Preparation of culture media

Potato Dextrose Agar (PDA) (Himedia M096) and Potato Dextrose Broth (PDB) were used in the antifungal bioassays while Muller-Hinton Agar (MHA) (Oxoid CMO337) and Muller-Hinton Broth (MHB) (Himedia M391) were used in antibacterial bioassay with Nutrient Agar (NA) (Himedia M001) being used for bacterial culture maintenance. The media were prepared by dissolving MHB in 1 liter, and 28 grams of NA in 1 litre. The media were mixed thoroughly by stirring until completely dissolved in water and then sterilized by autoclaving at 121°C and 15 psi pressure for 15 minutes. Potato dextrose broth was prepared as follows; potato infusion was prepared by boiling 200 g of sliced, unpeeled potatoes in 1 liter of distilled water for 30 min followed by filtering through a double layer cheesecloth and saving the effluent. The potato infusion was mixed with 20 grams of dextrose and autoclaved at

121°C and 15 psi pressure for 15 minutes. The final pH was adjusted to 5.6 and the medium stored in tightly closed bijou bottles. After autoclaving, the agar media was allowed to cool to 45°C and then dispensed into flat-bottomed Petri dishes (9cm in diameter) and allowed to solidify at room temperature (23 ± 2°C). Screw-capped bottles were used to store broth media. All the media were stored in a refrigerator until when required for use.

3.6.2 Retrieval of test pathogens and preparation of inocula

Stock culture for each of the test fungal pathogens maintained at -20°C was retrieved by sub-culturing on PDA. Three well-isolated pure colonies of the same morphological type were selected from an agar plate culture; a sterile loop was used to pick the top mycelia of each colony and the microbial was transferred into a test tube containing 10ml of PDB. The culture tubes were then incubated for 5 days at room temperature (23 ± 2°C) to obtain fresh fungal cultures. McFarland standard was used as a reference to adjust the turbidity of fungal suspensions to be within the required range. Exactly 0.5 McFarland equivalent turbidity standards was prepared by mixing 0.05ml of 1% barium chloride dehydrate with 9.95ml of 1% sulfuric acid. Fungal suspensions were prepared in sterile saline (0.85% NaCl wt/vol) prepared by dissolving 0.85g of NaCl in 100ml of distilled water and autoclaving for 15 minutes at 121°C and 15 psi pressure. The turbidity of the fungal suspensions was adjusted to 0.5 McFarland standard which is equivalent to 1×10^6 Colony Forming Units (CFU)/ml.

For the bacterial test pathogens, stock culture of each of the three bacteria maintained at -20°C was retrieved by sub-culturing on NA plates. Three well-isolated pure colonies of the same morphological type were selected from NA plates and aseptically transferred into test tubes containing 10ml of MHB using a sterile loop. The culture tubes were then incubated at 37°C for 24 hours to obtain fresh cultures. The turbidity of the bacterial suspensions for bioassays was adjusted to 0.5 McFarland standards; equivalent to 1.5×10^8 CFU/ml.

3.6.3 Preparation and sterilization of paper discs

Paper discs for use in the bioassays were prepared as follows; using a paper punch, paper discs 6mm in diameter were cut off from Whatman filter paper (No.1). The paper discs were then placed in a clean, dry, capped universal bottle and autoclaved

for 15 minutes at 121°C and 15 psi pressure. The paper discs were then stored in a sterile condition until when required for use.

3.6.4 Assessment of the antifungal activity of the essential oils

The antifungal activity of *T. minuta* essential oils against the test fungal pathogens was carried out using the disc diffusion method also known as Kirby-Bauer antimicrobial susceptibility test as described by Souza *et al*. (2005). The basic concept in this method is that, the size of the zone of inhibition can be correlated with the susceptibility of a microorganism to a particular antimicrobial agent. The diameter of the inhibition zones (mm) with zone margin taken as the area showing no apparent growth of the test pathogen judged by the naked eye is determined using a ruler. Since the diameter of the paper disc is 6 mm, it therefore, follows that 6 mm represent no inhibition.

A seven days old culture grown on PDA in the dark to promote sporulation was used to prepare a spore suspension which was adjusted to an optical density equal to 0.5 McFarland standards. One drop of Tween 20 per ml (approximately 0.01 to 0.02ml or 0.5 to 1%) was added as recommended by NCCLS (2002) to enhance the dispersion of spores. Two hundred microliters of the standardized spore suspension was uniformly spread using a sterile L-shaped glass rod on Petri plates (9cm in diameter) containing PDA medium. Sterile Whatman filter paper discs (No. 1, 6mm in diameter) were impregnated with 10µl of undiluted crude essential oil by holding the discs using a pair of sterile forceps and pipetting the EOs unto the paper discs using a micropipette. The discs were then aseptically placed at the center of the inoculated culture plates using a pair of forceps. Dimethyl sulphoxide was used as a negative control while Apron Star® (Thiamethoxam 200g/kg, Mefenoxam 200g/kg and Difenoconazole 20g/kg), a broad-spectrum seed treatment fungicide prepared according to the manufacturer's instruction was used as a positive control. The Petri dishes were then kept in a refrigerator at 4˚C for 2 hours to allow essential oils to diffuse into the agar medium. The plates were finally incubated at room temperature (23 ± 2°C) and the growth of the fungi monitored starting from the 3rd to 14th day. The reading of the diameters of the inhibition zones was done on the 5th and 10th day. This is because preliminary studies had shown that on the 3rd day, the fungi were still growing and between the 5th - 7th day, the oil activity was at

its highest. On the 14th day however, the oil activity against the test fungi was already decreasing, and there was overgrowth of the fungi. The tests were conducted in triplicates.

3.6.5 Assessment of the antibacterial activity of the essential oils

Antibacterial activity of *T. minuta* essential oils against the test bacterial phytopathogens was similarly carried out using the disc diffusion method. Two hundred microliters of a bacteria suspension prepared from an overnight culture was adjusted to an optical density equal to 0.5 McFarland and uniformly spread using a sterile L-shaped glass rod on Petri plates (9cm in diameter) containing MHA. Sterile Whatman filter paper discs (No. 1, 6mm in diameter) were each impregnated with 10µl of undiluted crude *T. minuta* essential oil in a sterile biological safety cabinet. The discs were then aseptically placed at the center of the inoculated culture plates using a sterile forceps. Dimethyl sulphoxide was used as a negative control while Enrich BM® (immunomodulator2-Bromo-2-Nitropropane-1,3 Diol), a broad-spectrum bactericide used in the control of bacterial diseases such as halo blight, bacterial wilt and bacterial spot was used as a positive control. The plates were refrigerated at 4˚C for 2 hours to allow the essential oils to diffuse into the agar medium and finally incubated upside down at 37°C for 48 hours. Measurement of the inhibition zones was after 24 hours and 48 hours. The tests were conducted in triplicates.

All the tests for antifungal and antibacterial activity of the essential oils against the test microorganisms were conducted in a biological safety cabinet and in accordance with the protocols of Clinical and Laboratory Standards Institute (CLSI) formerly National Committee for Clinical Laboratory Standards (NCCLS). For both the fungi and bacteria, the sensitivity of individual microorganisms to the essential oil was classified based on the inhibition zone values expressed as millimeters (mm) as follows: not sensitive (-) for total zone diameters equal to 8mm or below; sensitive (+) for diameters between 8 and 14mm; very sensitive (++) for zone diameters between 15 and 19mm and extremely sensitive (+++) for zone diameters equal to or larger than 20mm (Celikel and Kavas, 2008; Babu *et al.,* 2011).

3.6.6 Evaluation of antimicrobial activity of essential oil at different concentrations

The antimicrobial activity of different concentrations/dilutions of *T. minuta* essential oil against the test microorganisms was also studied. The activity of the EOs at seven concentration levels was evaluated using the disc diffusion method following the procedure described by Clara *et al*. (2013). Two hundred microliters of microbial suspension (approximately 10^6 and 10^8 CFU/ml for fungi and bacteria, respectively) was uniformly spread on PDA and MHA Petri plates for fungi and bacterial, respectively. Two-fold serial dilutions of *T. minuta* essential oil were prepared with pure DMSO as follows: A micropipette was used to dispense 500mL of DMSO in seven labelled and sterile Eppendorf tubes. Five hundred milliliters of crude essential oil was added to the first tube and the two liquids mixed using a micropipette. Five hundred milliliter was withdrawn from the contents of the first tube and transferred to the second tube and the content mixed using a micropipette. Five hundred milliliter was withdrawn from the contents of the second Eppendorf tube and transferred to the third tube. This process was repeated until 500mL of the content from tube 6 was withdrawn and subsequently added to tube 7. Thus, essential oils of 50%, 25%, 12.5%, 6.25%, 3.13%, 1.56% and 0.78% concentrations were prepared.

Sterile Whatman filter paper discs (No. 1, 6mm in diameter) were impregnated with 10µl of different essential oil concentrations and aseptically placed at the center of the inoculated culture plates. The plates were then placed in a refrigerator at 4˚C for 2 hours to allow the essential oils to diffuse into the agar. The plates were then incubated at room temperature (23 ± 2°C) for fungi and at 28°C for 48 hours for bacteria. The diameters of the inhibition zones were then measured after 5 days for fungi and 48 hours for bacteria. The tests were conducted in triplicates and in addition to evaluating the activity of different concentrations of essential oil against the test microorganisms, this test was also used to estimate the minimum inhibition concentrations (MICs) of the oil on the test microorganisms (Clara *et al*., 2013).

3.6.7 Minimum inhibitory concentrations and minimum bactericidal/fungicidal concentrations

Tube dilution method as described by Caburian and Osi (2010) with some modifications was employed in the evaluation of minimum inhibitory concentrations (MICs), minimum bactericidal concentration (MBCs) and minimum fungicidal concentration (MFCs) of the essential oils against the test microorganisms. Twelve sterile screw-capped falcon tubes (15ml) were numbered #1 to #11 and the last one as #13. One milliliter of Muller-Hinton/Potato dextrose broth was introduced into tubes #2 to #11. One milliliter of *T. minuta* essential oil was pipetted into tube #1 and #2, the two tubes were capped and vortexed for 5 seconds; one milliliter was withdrawn from the contents of tube #2 and transferred to tube #3, after capping the tube and mixing by shaking the contents, one milliliter from the contents of tube #3 was withdrawn and transferred to tube #4, the tube was capped, shaken and mixed well. This process was repeated until 1.0ml of the content from tube #9 was withdrawn and subsequently added to tube #10, capped and shaken. Fifty microliters of standardized inocula (approximately $10^6$and 10^8 CFU/mL for fungi and bacteria, respectively) was then introduced into tubes #1 to #11 and to tube #13. To tube #13, 0.5ml of a standard bactericide/fungicide prepared according to the manufacturer's instructions was added. Tween 20 (0.05%) was added to the essential oil prior to its application into the tubes to improve the oil solubility and also for better spore dispersion in the case of fungi (NCCLS, 2002). The tubes were then incubated at 37°C for 48 hours for bacteria and 25°C for 72 hours for fungi.

In summary, for this bioassay test, all the tubes numbered #1 contained inoculum and undiluted crude essential oil; tubes #2 - #10 contained broth medium, inoculum and essential oil of decreasing concentrations (380mg/ml – 1.5mg/ml); tube #11 contained the inoculum, broth medium but no essential oil and thus acted as a negative control; tube #13 contained the inoculum and a standard commercial fungicide/bactericide and thus acted as a positive control.

After incubation, the tubes were examined for growth by observing for any turbidity. The interest here was not on the dilutions that had failed to prevent microbial growth but rather, those that prevented any visible growth. The concentration in the tube with the lowest concentration (highest dilution) of the essential oil at which no visible growth or turbidity was observed was reported as the minimum inhibitory concentration of the oil on the test microorganism (Quinto and Santos, 2006). The tubes were then shaken to homogenize the

contents and 0.01ml of the contents of each tube taken and subcultured by streaking on potato dextrose agar and Mueller-Hinton agar plates for fungi and bacteria, respectively. The plates were then inverted and incubated at 37°C for 24 hours in the case of bacteria while for fungi, the plates were incubated at room temperature (23 ± 2°C) for 72 hours and then observed for any growth of colonies. Minimum fungicidal/bactericidal concentration was determined as the highest dilution (lowest concentration) of essential oil at which no growth occurred following the subculturing onto PDA and MHA plates within the specified incubation periods (Quinto and Santos, 2006). The minimum inhibitory concentration values obtained using the tube dilution method for all the tested microorganisms were compared with those obtained using the disc diffusion method for any similarities or differences.

3.7 Characterization of chemical composition of the essential oils of *Tagetes minuta*

The chemical composition of the essential oils of *Tagetes minuta* was established by gas chromatography (GC) coupled to mass spectrometry (MS) at the International Centre of Insect Physiology and Ecology (ICIPE), Nairobi. Three replicates (each taken from a different extraction batch) of 1mg of *T. minuta* essential oils were separately weighed and diluted in 1ml volume of dichloromethane to make a stock solution. From the stock solution, further dilution was made as follows, 100µl of stock topped to 1ml with dichloromethane after which they were analyzed on an HP-7890A (Agilent Technologies, Wilmington, USA) GC connected to an HP 5975 C (Agilent Technologies, Wilmington, USA) MS. The gas chromatography equipment was fitted with HP-5MS capillary column; 30m × 0.25mm internal diameter; 0.25µm film thickness with 5%-phenyl methyl silicone as the stationary phase (J & W Scientific, Folsom, USA). The carrier gas was Helium (1.2 ml min^{-1}); with the oven temperature programmed at 35 °C (for 5 min) to 280 °C at 10 °C min^{-1} and then held isothermal at 280°C for 10.5 min.; injection mode was splitless. Mass spectra were acquired at 70 eV within a mass range of 38–550 Daltons (Da) with a scan time of 0.73 scans per second whereas the ion source was maintained at 230 °C. Identification of the essential oil components was achieved on the basis of their retention indices (RI) (determined with reference to a homologous series of normal alkanes C_5-C_{31}) and calculated based on the equation of Van den Dool and Kratz shown below and compared with what is documented in literature (Van den Dool and Kratz, 1963; Adams, 2007; Derwich *et al*., 2010).

$$RIx = 100\, n_0 + 100\, (R_Tx - R_Tn_0) / (RTn_1 - RTn_0)$$

Where:

x the name of the target compound

n_0 n-alkane Cn_0H2n_0+2 directly eluting before x

n_1 n-alkane Cn_1H2n_1+2 directly eluting after x

R_T retention time

RI retention index

The identity of the essential oil constituents was further verified by comparison of their mass spectral fragmentation patterns with those reported in the mass spectra with library data (NIST05a and Adams MS HP, USA). To quantify terpenes in the essential oil, serial dilutions of authentic standard (1, 8-cineole; 99%, Gillingham, Dorset, England) (50- 550ng/µl) were analyzed by GC/MS in full scan mode to generate a linear calibration curve (peak area vs. concentration) with the following equation:

$$y = 7E{+}06x - 1E{+}07 \quad (R^2{=}0.9736)$$

Where:

y peak area

x concentration

E Exponential

This was used for external quantification of different terpenes with the relative amount of each individual component expressed as percentages of the peak area relative to the total peak area.

3.8 Data analysis

Data on inhibition of test pathogens were analyzed using the PROC ANOVA procedure of GENSTAT version 15 and significant differences amongst means compared using Fisher's Protected LSD at 5% probability level. The inhibitory effects of the essential oils against the test pathogens were expressed as mean ± standard error of the mean inhibition zones diameter (mm). Linear regression analysis was performed to find correlations between different concentrations of essential oil and their overall antimicrobial activity assessed as diameter of inhibition with regard to all the tested fungi and bacteria species. Standard dose-response curves were obtained by plotting essential oil concentrations (mg/ml) against the mean inhibition zone diameters (mm).

CHAPTER FOUR: RESULTS

4.1 Morphological features of *Tagetes minuta*

The summary of the morphological features of the sampled *Tagetes minuta* plants is shown in Figure 3 (Authors' field observations). The leaves of the plants were slightly glossy to light green in colour, glabrous, 7-14 cm long and pinnately compound dissected into 4 to 6 pairs of pinnae. The leaflets were linear, glabrous and with visible sunken oil glands. On the undersurface of the leaves, small, multicellular and punctate glands which were orangish in colour were visible and when punctured, these glands produced liquorice-like aroma. The heads were small; 10-15mm long, and including ray florets, with each head surrounded by 4 to 5 fused involucre bracts. The heads were borne in a clustered panicle of 20 to 80 capitula.

Figure 3: Morphological characteristics of *Tagetes minuta* L.

(A) Habit, (B) Leaf under surface, (C) Leaf upper surface, (D) Heads (E) Leaflet.

4.2 Percentage yield of *Tagetes minuta* essential oils

Four distillation batches of approximately 4kg of leaves, flowers and stems of *T. minuta* plant material were carried out. Table 1 shows that, 10.28 grams of *T. minuta* essential oils equivalent to 13.53ml was obtained from 17.3 kilograms of plant material. A mean yield of 0.059% w/w of *T. minuta* essential oil was obtained from the four distillation batches. The standard deviation of the four extraction batches yield (% w/w) was found to be 0.0006 which

is equivalent to 1.01% relative standard deviation (RSD). The % of RSD showed that the steam distillation process was precise with minimal wastages.

Table 1: Percentage yield (% w/w) of *Tagetes minuta* essential oil

Batch No.	Weight of plant material (kg)	Weight of essential oil (g)	Percentage yield (% w/w)
1	4.38	2.588	0.059
2	4.23	2.542	0.060
3	4.60	2.738	0.059
4	4.10	2.412	0.058
		Mean =	0.059 ± 0.0003

4.3 Physical and chemical characteristics of *Tagetes minuta* essential oil

Tagetes minuta essential oils obtained were less dense and insoluble in water. The EOs were however soluble in ethanol, DMSO and CH_2Cl_2 at a level of 1:1(v/v). The oils exhibited a pale yellowish-orange colour (Figure 4) with a citrus-like and turpentine-like odour. The oils were liquid at room temperature (23 ± 2°C) and maintained this state even in storage a -20°C. However, when mixed with DSMO in all the dilution levels used in the study, the essential oils froze when stored at -20°C. The essential oils had a density of 0.76g/ml.

Figure 4: *Tagetes minuta* essential oil extracted from samples collected in Maseno, Kisumu County exhibiting a pale yellow-orange colour

4.4 Activity of *Tagetes minuta* essential oil on selected fungal pathogens

Tagetes minuta essential oils were found to have activity against all the tested fungal pathogens (Figure 5; Figure 6). In addition to the observed growth inhibition as revealed by the inhibition zone diameters of different sizes, the essential oils were shown to cause a delay in the growth of *A. niger*, *A. parasiticus* and *A. flavus* as shown in Figures 5 and 6.

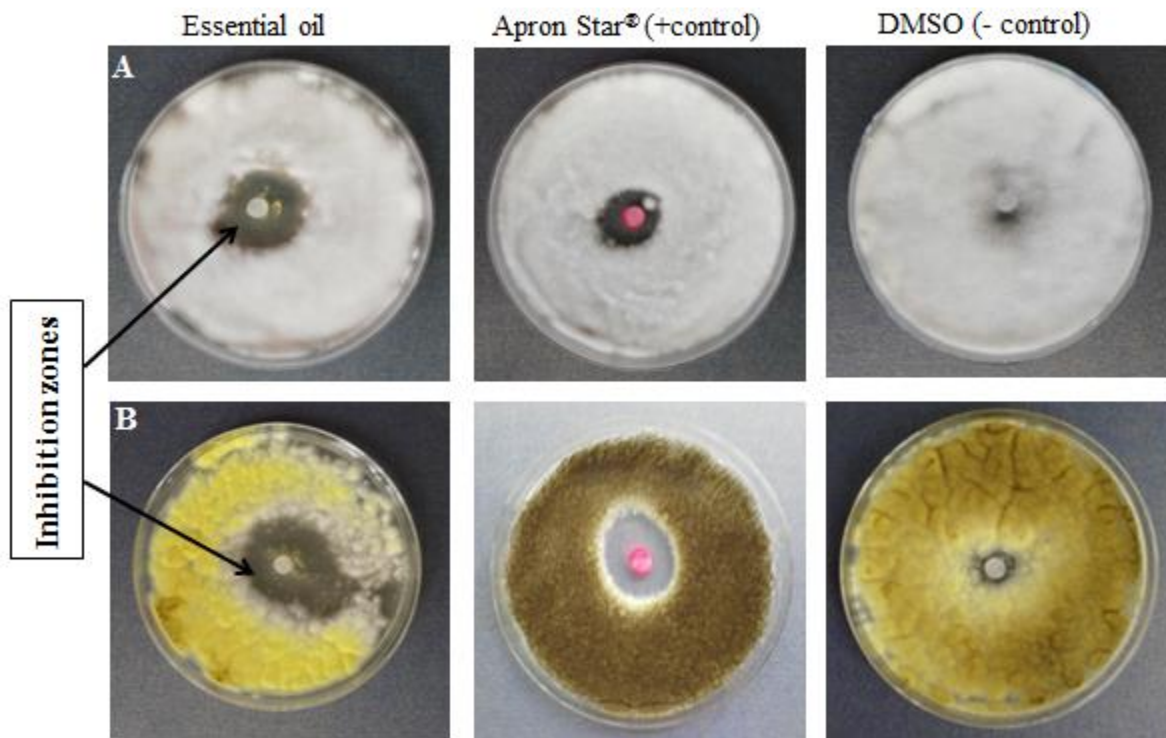

Figure 5: Inhibition zones of essential oils of *Tagetes minuta* on (A) *F. oxysporum* and (B) *A. niger* and controls after 10 days

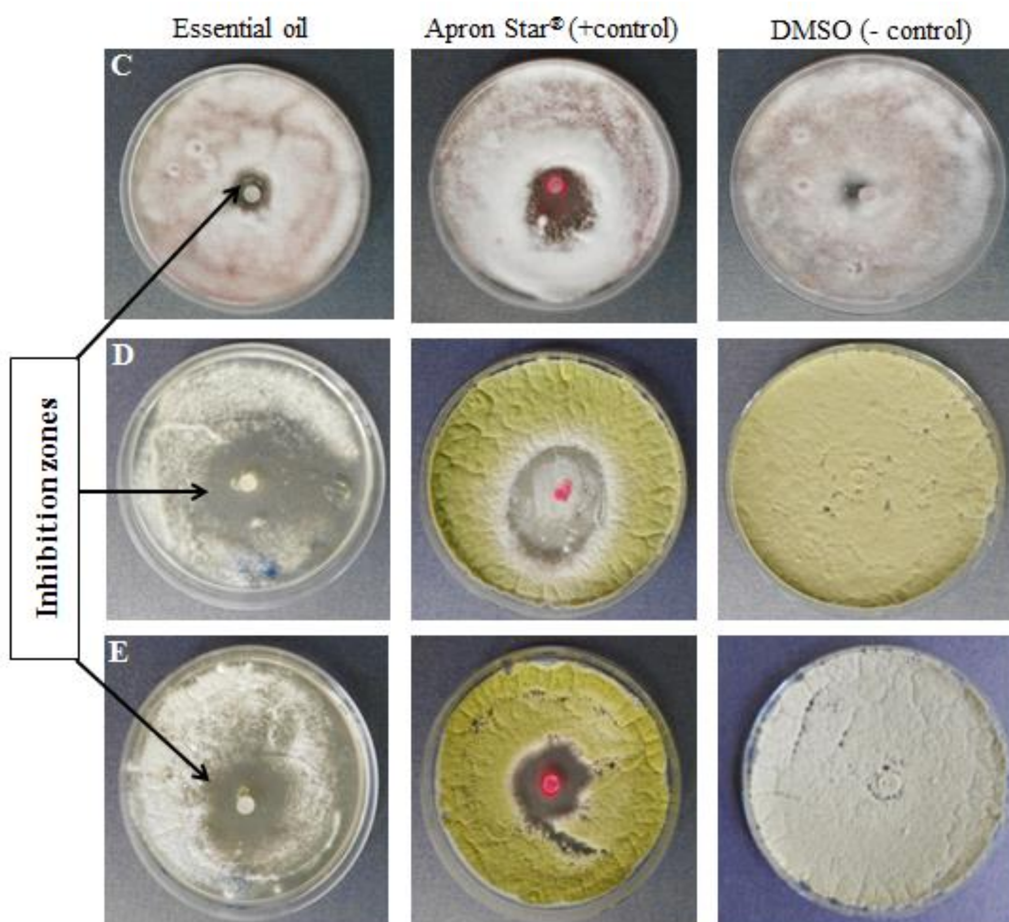

Figure 6: Inhibition zones of essential oils of *Tagetes minuta* on (C) *F. solani,* (D) *A. parasiticus* and (E) *A. flavus* and controls after 10 days

The results revealed that on the 5th day the highest antifungal activity of the oils was agaisnt *F. oxysporum* and *A. niger,* both of which had mean inhibition zone diameters of 28.67mm (Table 2). The oils activity against these two fungal species was significantly greater ($p \leq 0.05$) than that observed from the standard fungicide Apron Star® within the same period. The standard fungicide produced mean inhibition zones of 20.33mm and 24.50mm in *F. oxysporum* and *A. niger,* respectively after a 5 day incubation period. On the 10th day, the oils produced inhibiton zones of 22.67 and 16.83mm in *F. oxysporum* and *A. niger*, resepctively. These values were significantly lower ($p \leq 0.05$) in comparison to those recorded on the 5th day.

In general, the activity of the oils against the five tested fungal species on the 10th day was significantly lower ($p \leq 0.05$) compared to the activity observed on the 5th day. The

percentage decrease in the inhibition zones within the two period was 29%, 21%, 18%, 41% and 14% for *F. solani, F. oxysporum, A. flavus, A. niger* and *A. parasiticus*, respectively. Additonal details on the analysis of variance for the inhibition zones produced by *T. minuta* essential oil and Apron Star® on the five test fungi after the 5th and 10th day are shown in Appendix A.

Table 2: Inhibition zones (mm) of *Tagetes minuta* essential oils and Apron star® on five fungi after five and ten days of incubation

Fungi	*Tagetes minuta* essential oil inhibition zones (mm)		Apron Star® inhibition zones (mm) (Positive Control)		Mean
	5th day	10th day	5th day	10th day	
F. solani	19.83±0.60[ef]	14.17±0.83[h]	21.83±0.44[c-e]	19.17±0.44[fg]	18.75
F. oxysporum	28.67±0.88[a]	22.67±0.88[b-d]	20.33±1.20[d-f]	19.00±0.58[fg]	22.67
A. flavus	23.17±0.44[bc]	19.00±1.15[fg]	22.17±1.30[b-e]	23.33±0.88[bc]	21.92
A. niger	28.67±1.20[a]	16.83±0.73[f]	24.50±0.76[b]	24.00±0.58[bc]	23.50
A. parasiticus	28.23±0.39[a]	24.17±0.60[bc]	28.33±0.88[a]	30.50±1.04[a]	27.81

Values are mean ± standard error of the mean for bioassay conducted in triplicates. Means followed by the same alphabetical letter(s) within columns and across rows are not significantly different (Multivariate analysis, Fisher's protected LSD at $p \leq 0.05$).

While *F. oxysporum* was the most susceptible to the activity of the essential oils among all the test fungi, *F. solani* was the least sensitive to the essential oils as was indicated by it having the smallest mean inhibition zones of 19.83 and 14.17mm on the 5th and 10th day, respectively. Comparison of the antifungal activity of the essential oils and the standard fungicide on the five test fungi after 5 and 10 days of incubation are shown in Figure 7A and Figure 7B, respectively.

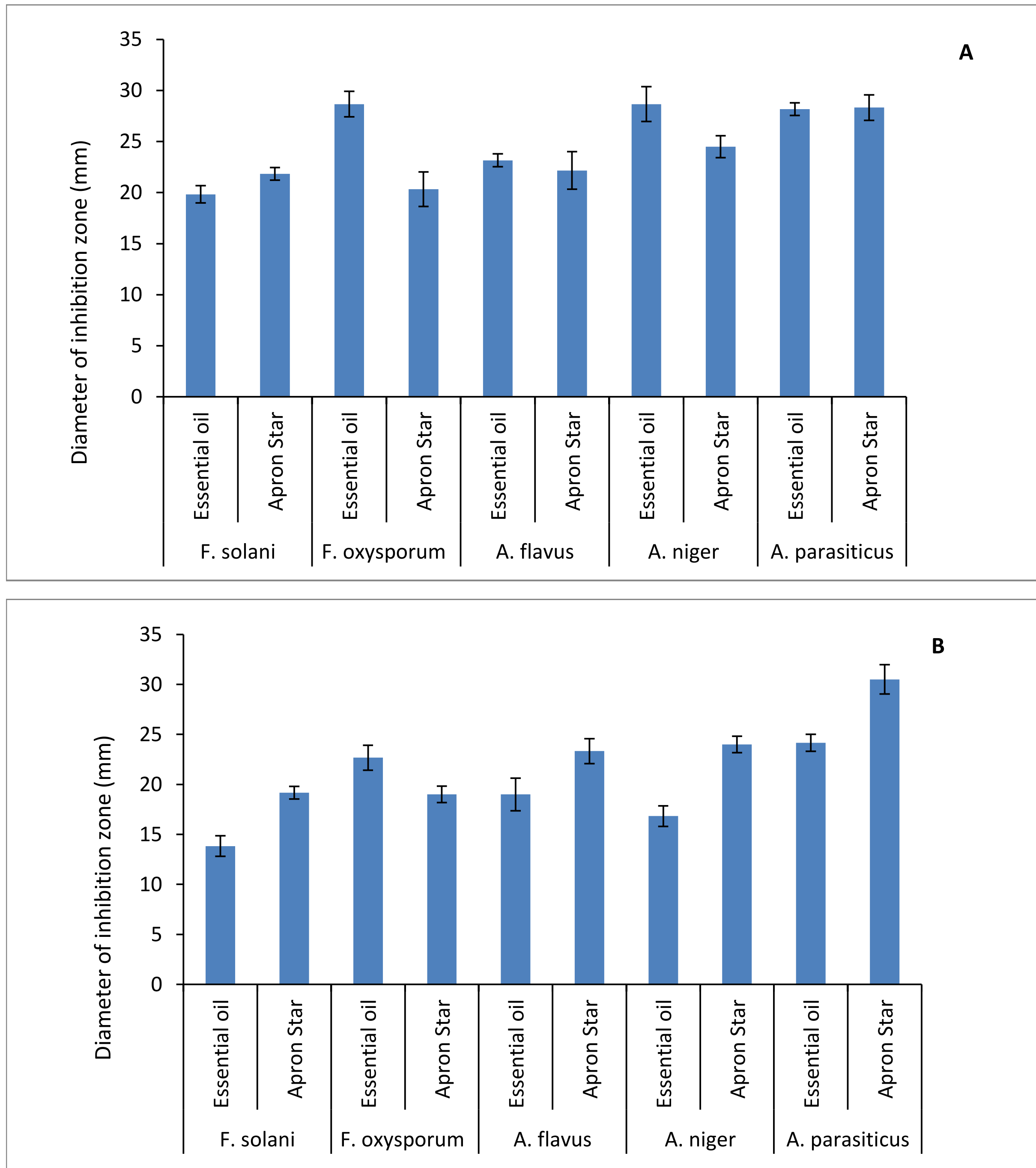

Figure 7: Growth inhibition of the test fungi by *Tagetes minuta* essential oils and Apron Star® after (A) 5 days and (B) 10 days of incubation

Error bars represent standard error of the mean.

4.5 Activity of different concentrations of *Tagetes minuta* essential oil on selected fungi

The antifungal activity of the essential oils was concentration-dependent and the activity of the oils at different concentrations varied among the fungal species (Table 3). Generally, as the concentration of the essential oil increased, the activity against the test fungi increased too. Thus, sizes of the inhibition zones produced were directly proportional to the concentration/dilution levels of the essential oil with the inhibition zone sizes decreasing with decrease in the oil concentration. There were however, a number of exceptional cases where more diluted oils produced larger inhibition zones than less diluted oils. The highest activity of the oil was observed on *A. parasiticus* which had an inhibition zone of 22.83mm at an oil concentration of 380mg/ml after 5 days. The essential oils at concentrations of 190, 95 and 48mg/ml produced inhibition zones of 20.17, 14.67 and 10.33mm, respectively in this fungus. The last three oil concentrations i.e. 24, 12 and 6mg/ml did no produce any appreciable inhibition zones (i.e. > 8.00mm).

Fusarium oxysporum similarly showed different levels of susceptibility at different EO concentrations. At an oil concentration of 380mg/ml, an inhibition zone of 20.83mm was produced, while inhibition zones of 18.17mm, 19.33mm and 19.33mm were produced at oil concentrations of 190mg/ml, 95mg/ml and 48mg/ml respectively with 12mg/ml being the lowest concentration of the oil at which a substantial inhibition zone was recorded. The result for *F. oxysporum* additionally revealed that the relationship between mean zone of inhibitions and the oil concentrations was not a perfect one as was the case with *A. parasiticus*. For example, inhibition zones of 19.33mm were obtained at 95mg/ml and 48mg/ml, while at 190 mg/ml, a mean inhibition zone of 18.17mm was recorded. This was one of the examples where a less concentrated essential oil resulted in a large inhibition zone than more concentrated oil. Based on this bioassay, the highest concentrations of the essential oil at which no appreciable inhibition zones were observed were; 24mg/ml (*F. solani*), 6mg/ml (*F. oxysporum*), 24mg/ml (*A. flavus*), 6mg/ml (*A. niger*) and 24mg/ml (*A. parasiticus*). These values are the minimum inhibitory concentrations (MICs) for the five test fungal species based on the disc diffusion method.

Table 3: Inhibition zones (mm) on test fungi by different concentrations of *Tagetes minuta* essential oils after five days of incubation

Fungi species	Essential oil concentration (mg/ml)*10^2							DMSO (-control)
	3.8	1.9	0.95	0.48	0.24	0.12	0.06	
F. solani	13.50±0.76	14.83±2.49	9.50±10.76	8.50±0.50	≤8.00	≤8.00	≤8.00	0.00
F. oxysporum	20.83±0.93	18.17±0.60	19.33±0.17	19.33±0.44	12.50±0.87	10.17±0.44	≤8.00	0.00
A. flavus	18.83±0.44	19.50±0.76	13.83±1.09	8.67±0.44	≤8.00	≤8.00	≤8.00	0.00
A. niger	19.33±0.40	19.17±0.60	21.33±0.60	13.83±1.09	12.83±0.73	9.17±0.43	≤8.00	0.00
A. parasiticus	22.83±0.44	20.17±0.93	14.67±0.93	10.33±0.60	≤8.00	≤8.00	≤8.00	0.00

Values are means ± standard error of the mean for bioassay conducted in triplicates

The result of the antifungal activity of different concentrations of the essential oil showed a dose-response correlation between the oil concentration and the inhibition of fungal growth represented by the inhibition zone diameters. A linear regression model based on essential oil concentration (mg/ml) as the input independent variable for the test fungi each analyzed separately is shown in Figure 8. There was a significant correlation ($p \leq 0.05$) between the tested concentrations of the essential oils and mean inhibition zones in *A. parasiticus* (R^2=0.87; p=0.002), *A. flavus* (R^2=0.72; p=0.009) and *F. solani* (R^2 = 0.73; p=0.014). An exception to this pattern was however in *A. niger* (R^2=0.36; p=0.092) and *F. oxysporum* (R^2=0.35; p=0.096) where no significant correlation ($p \leq 0.05$) was found between the tested essential oil concentrations and the mean inhibition zones. Further estimates of parameters for this linear regression analysis are summarized in Appendix B.

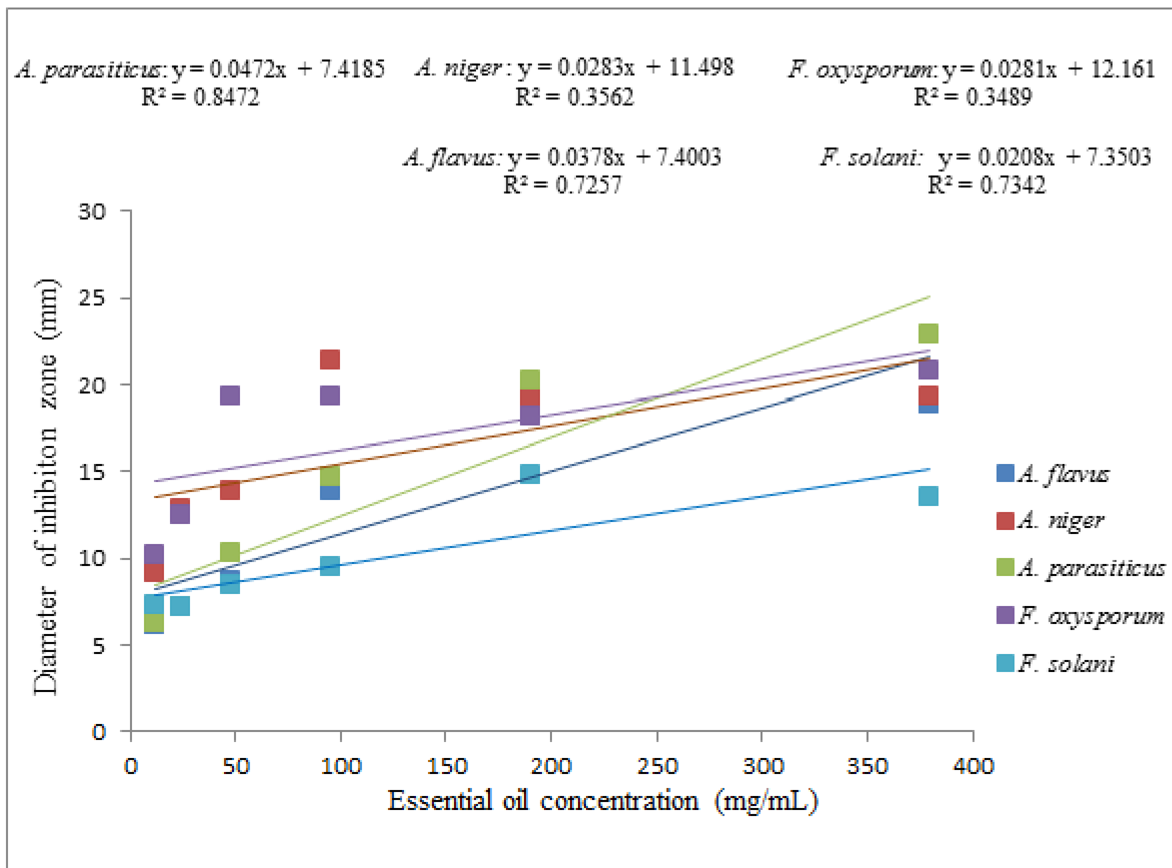

Figure 8: A dose-response curve of inhibition zone diameters (mm) against the concentration of *T. minuta* essential oil (mg/ml) for the five test fungi.

4.6 Minimum inhibitory concentrations and minimum fungicidal concentrations

The results of the minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) of *T. minuta* essential oils on the five tested fungi are shown in Table 4. The minimum inhibitory concentrations of the oil ranged from 24 to 95mg/ml. In *F. oxysporum*, *A. flavus* and *A. niger*, the lowest concentration of the oil at which no visible growth was detected in PDB tubes after incubation was 24mg/ml which was thus the MICs of the essential oils on the three fungal species. The lowest concentrations of the oils at which no growth occurred on PDA plates were 190mg/ml, 48 mg/ml and 24mg/ml for *F. oxysporum*, *A. flavus* and *A. niger*, respectively. These values were taken as the minimum fungicidal concentrations for the three respective fungi. The results also revealed that the three aforementioned fungi had the lowest MICs values of all the five test fungi i.e. 24mg/ml. The minimum fungicidal concentration of the oil against the test fungi ranged from 24 to 190mg/ml with the lowest and highest MFCs value observed in *A. niger* and *F. solani*, respectively. In the case of *A. niger*, the minimum inhibitory concentration and minimum fungicidal concentration values were the same i.e. 24mg/ml. *Aspergillus parasiticus* similarly had the same minimum inhibitory concentration and minimum fungicidal concentration value i.e. 48mg/ml.

Table 4: Minimum inhibitory concentrations and minimum fungicidal concentrations of the essential oils of *Tagetes minuta* on five fungal species

Tube/Plate No.	EOs concentration (mg/ml)*10^2	Growth of fungi in PDB tubes					Growth of fungi on PDA plates				
		FS	FO	AF	AN	AP	FS	FO	AF	AN	AP
1	Crude EOs	-	-	-	-	-	-	-	-	-	-
2	3.8	-	-	-	-	-	-	-	-	-	-
3	1.9	-	-	-	-	-	-	-	-	-	-
4	0.95	-	-	-	-	-	+	-	-	-	-
5	0.48	+	-	-	-	-	+	-	-	-	-
6	0.24	+	-	-	-	+	+	+	+	-	+
7	0.12	+	+	+	+	+	+	+	+	+	+
8	0.06	+	+	+	+	+	+	+	+	+	+
9	0.03	+	+	+	+	+	+	+	+	+	+
10	0.015	+	+	+	+	+	+	+	+	+	+
11	- ve control	+	+	+	+	+	+	+	+	+	+
13	+ve control	-	-	-	-	-	-	-	-	-	-

[+] Growth and [-] No growth of the fungi; FS - *F. solani*, FS - *F. oxysporum*, AF - *A. flavus*, AN - *A. niger* and AP - *A. parasiticus*

PDA - Potato dextrose agar
PDB- Potato dextrose broth
Tube 1- Fungal inoculum and undiluted crude essential oils
Tube 2 to 10 - PDB, fungal inocula and essential oils of different concentrations
Tube 11- Fungal inoculum and PDB (-ve control)
Tube 13- Fungal inoculum, PDB and the standard fungicide (+ve control)

4.7 Activity of *Tagetes minuta* essential oil against selected phytopathogenic bacteria

In vitro studies demonstrated the antibacterial activity of *Tagetes minuta* essential oils against the three tested plant pathogenic bacteria viz., *Pseudomonas savastanoi* pv. *phaseolicola* (PSP), *Xanthomonas axonopodis* pv. *phaseoli* (XAP) and *Xanthomonas axonopodis* pv. *manihotis* (XAM) as shown in Figure 9.

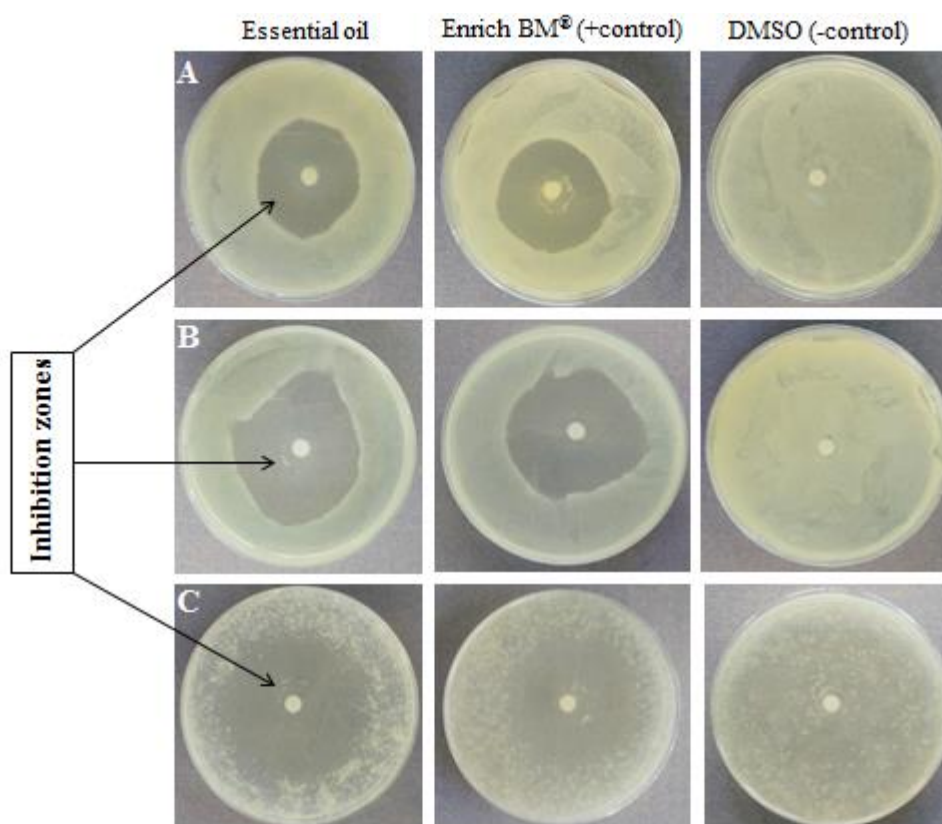

Figure 9: Inhibition zones (mm) of *Tagetes minuta* essential oil against (A) *X. axonopodis* pv. *manihotis*, (B) *X. axonopodis* pv. *phaseoli*, (C) *P. savastanoi* pv. *phaseolicola* and controls after 48 hours.

The inhibitory activity of the essential oils and hence the inhibition zone diameters differed among the three test bacteria. *Pseudomonas savastanoi* pv. *phaseolicola* had the highest susceptibility to the oil with mean inhibition zone diameters of 41.83 and 44.83mm after an incubation period of 24 and 48 hours, respectively. The inhibition zone produced by *T. minuta* essential oil on *P. savastanoi* pv. *phaseolicola* was significantly higher ($p \leq 0.05$) than that of the standard bactericide Enrich BM®, both after 24 and 48 hours of incubation (Table 5). The essential oil produced inhibition zones of 26.83mm in both *X. axonopodis* pv.

manihotis and *X. axonopodis* pv. *phaseoli* after 24 hours. Furthermore, while the antimicrobial activity of the essential oils against *X. axonopodis* pv. *phaseoli* increased significantly after another 24 hours, the activity of the oil against *X. axonopodis* pv. *manihotis* decreased significantly within the same period ($p \leq 0.05$).

Overall, the essential oil exhibited maximum activity in *X. axonopodis* pv. *manihotis* after 24 hours of incubation, while for *P. savastanoi* pv. *phaseolicola* and *X. axonopodis* pv. *phaseoli*, the highest activity of the oil occurred after 48 hours. Furthermore, after 24 hours, the activity of the oil against *P. savastanoi* pv. *phaseolicola* was significantly higher ($p \leq 0.05$) to that shown in *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *manihotis*. However, the antimicrobial activity of the essential oil against the latter two bacterial test pathogens was not significantly different ($p \leq 0.05$). The percentage change in the inhibition zones produced by the essential oil on the test bacteria between 24 and 48 hours was 3%, 14% and 7% for *X. axonopodis* pv. *phaseoli, X. axonopodis* pv. *manihotis* and *P. savastanoi* pv. *phaseolicola*, respectively. Further details on the analysis of variance for the inhibition zones produced by *T. minuta* essential oil and Enrich BM® on the test bacteria after 24 and 48 hours are given in Appendix C.

Table 5: Inhibition zones (mm) of *Tagetes minuta* essential oils and Enrich BM® on three test bacteria pathogens after 24 and 48 hours

Bacteria species	24 hours			48 hours		
	Essential oil	Enrich BM® (+ve control)	DMSO (-ve control)	Essential oil	Enrich BM® (+ control)	DMSO (-ve control)
XAP	26.83 ± 0.60^{f}	29.50 ± 0.29^{e}	0.00	27.67 ± 0.88^{ef}	29.50 ± 0.29^{e}	0.00
XAM	26.83 ± 0.17^{f}	35.17 ± 0.73^{cd}	0.00	23.00 ± 1.00^{g}	37.00 ± 0.58^{c}	0.00
PSP	41.83 ± 0.93^{b}	33.17 ± 0.93^{d}	0.00	44.83 ± 0.83^{a}	33.17 ± 0.73^{d}	0.00
Mean	31.83	32.61		31.83	33.22	

Values are mean ± standard error of the mean for bioassay conducted in triplicates. Means followed by the same letter(s) within columns and across rows after 24 and 48 hours incubation are not significantly different (Multivariate analysis, Fisher's protected LSD at $p \leq 0.05$).
XAP- *Xanthomonas axonopodis* pv. *phaseolicola*
XAM- *Xanthomonas axonopodis* pv. *manihotis*
PSP- *Pseudomonas savastanoi* pv. *phaseolicola*

The standard bactericide that was used as a positive control showed the highest antimicrobial activity against *X. axonopodis* pv. *manihotis* producing mean inhibition zones of 35.17 and 37.00mm after 24 and 48 hours, respectively. The difference in the activity of the bactericide against *X. axonopodis* pv. *manihotis* within the two periods was significantly different ($p \leq 0.05$). With regard to *X. axonopodis* pv. *phaseoli* and *P. savastanoi* pv. *phaseolicola* however, the antimicrobial activity of Enrich BM® within the two specified periods was not significantly different ($p \leq 0.05$). Generally, *P. savastanoi* pv. *phaseolicola* and *X. axonopodis* pv. *manihotis* showed the highest and lowest susceptibility to the essential oils, respectively . Figure 10A and Figure 10B show a comparison of the antibacterial activity of *T. minuta* essential oils and Enrich BM® on the test bacteria after a 24 and 48 hours, respectively.

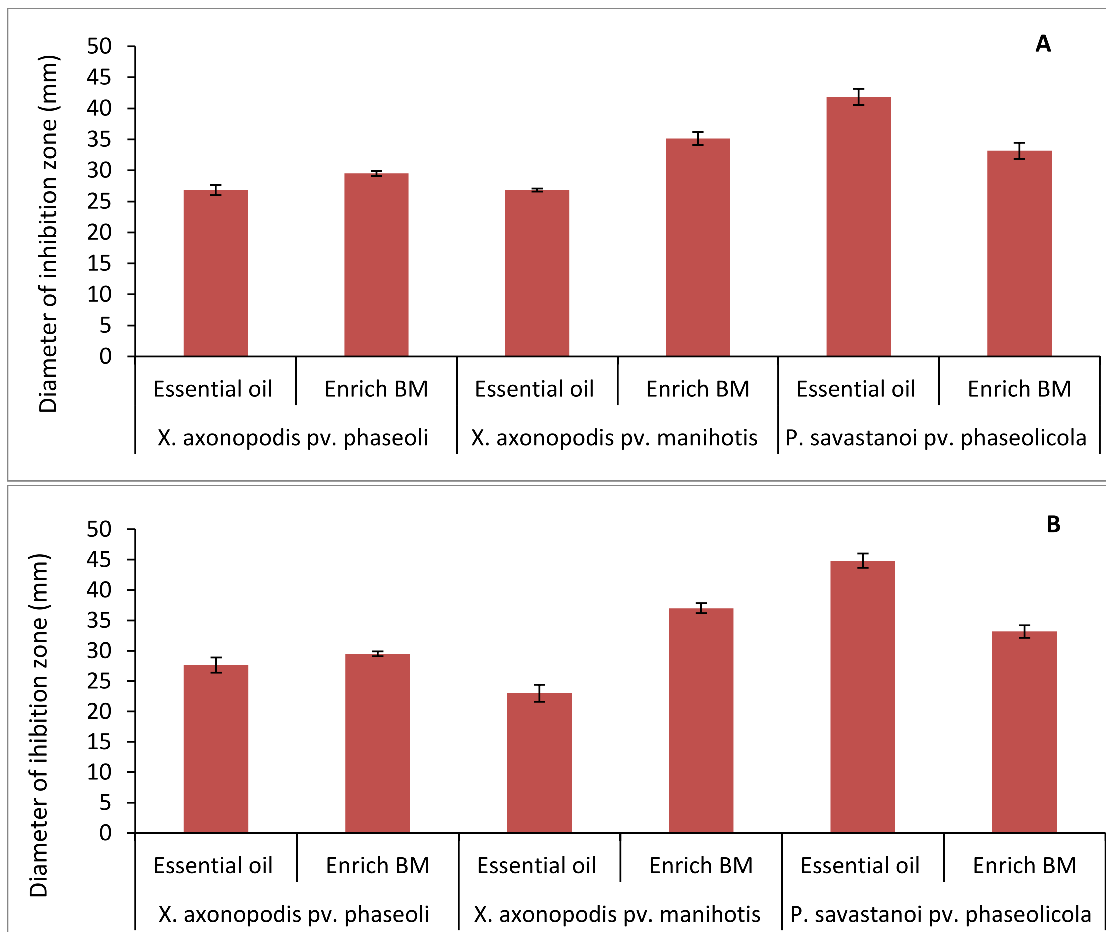

Figure 10: Growth inhibition of the test bacteria by *Tagetes minuta* essential oils and Enrich BM® after (A) 24 hours and (B) 48 hour of incubation

Error bars represent standard error of the mean**.**

4.8 Activity of different concentrations of *Tagetes minuta* essential oil on selected bacteria

The antibacterial activity of *T. minuta* essential oils was concentration-dependent. Thus, as the concentration of the essential oils increased, the activity of the oils against the test bacteria increased (Table 6). The results furthermore revealed that the inhibition zones produced at different essential oil concentrations varied form one bacterial species to another. The highest activity of the oil was observed in *P. savastanoi* pv. *phaseolicola* with inhibition zones of 21.33 and 17.67mm at 380 and 190mg/ml essential oil concentrations, respectively. The highest concentration of EO at which no appreciable inhibition zone was recorded for *P. savastanoi* pv. *phaseolicola* was 12mg/ml, which was taken as the minimum inhibitory concentration (MIC) of the essential oil on this bacterium based on the disc diffusion assay method. Among the three test bacteria, the essential oils had the lowest activity on *X. axonopodis* pv. *manihotis* where inhibition zones of 12.83, 10.83, and 10.50mm were produced at concentrations of 380, 190 and 95mg/ml, respectively. The highest concentrations of EO at which no appreciable inhibition zones were recorded (i.e. MICs) for *X. axonopodis* pv. *manihotis* and *X. axonopodis* pv. *phaseolicola* were 48 and 24mg/ml, respectively.

Table 6: Inhibition zones (mm) of three bacterial species at different concentrations of *Tagetes minuta* essential oil after 48 hours

Bacteria species	Essential oil concentration (mg/ml)*10^2							DMSO (-ve control)	MIC (mg/ml)
	3.8	1.9	0.95	0.48	0.24	0.12	0.06		
XAP	17.33±0.60	13.67±1.01	11.17±0.60	8.17±0.17	≤8.00	≤8.00	≤8.00	0.00	24.00
XAM	12.83±0.60	10.83±0.93	10.50±0.29	≤8.00	≤8.00	≤8.00	≤8.00	0.00	48.00
PSP	21.33±0.44	17.67±0.17	13.17±0.60	9.83±0.16	8.17±0.17	≤8.00	≤8.00	0.00	12.00

Values are means ± standard error of the mean for bioassay conducted in triplicates.

XAP - Xanthomonas *axonopodis* pv. *phaseolicola*
XAM - *Xanthomonas axonopodis* pv. *manihotis*
PSP - *Pseudomonas savastanoi* pv. *phaseolicola*
MIC - Minimum inhibitory concentration

The results on regression analysis for the relationship between the diameters of the inhibition zones (mm) and concentrations of *T. minuta* essential oils (mg/ml) are shown in Figure 11. The dose-response model revealed that there was a significant correlation ($p \leq 0.05$) between the concentrations of *T. minuta* essential oils and the mean inhibition zones for the three tested bacteria namely; *X. axonopodis* pv. *phaseoli*, *P. savastanoi* pv. *phaseolicola* and *X. axonopodis* pv. *manihotis.* The correlation coefficient values obtained for these bacterial species were: R^2, 0.94, p= <0.001; R^2, 0.91, p= <0.001, and R^2, 0.84, p= 0.004, respectively. These results thus indicated that 94, 91 and 84% of the variation in the diameters of the inhibition zones in *X. axonopodis* pv. *phaseoli*, *P. savastanoi* pv. *phaseolicola* and *X. axonopodis* pv. *manihotis*, respectively was explained by the variation in the concentration of the essential oils. Further estimates of parameters for this linear regression analysis are shown in Appendix D.

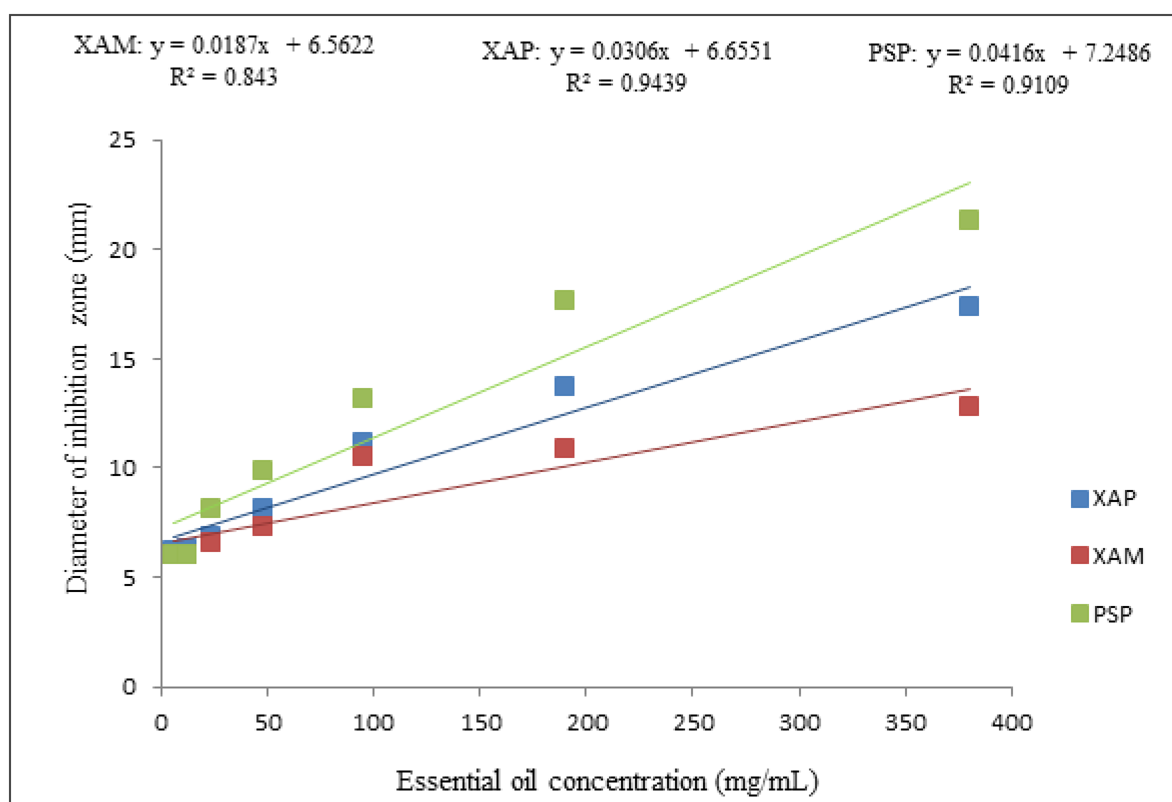

Figure 11: A dose-response curve of inhibition zone diameters (mm) against the concentration of *T. minuta* essential oil (mg/ml) for the test bacteria.

XAP - *Xanthomonas axonopodis* pv. *phaseolicola*
XAM - *Xanthomonas axonopodis* pv. *manihotis*
PSP - *Pseudomonas savastanoi* pv. *phaseolicola*

4.9 Minimum inhibitory concentrations and minimum bactericidal concentrations

The results on the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of *Tagetes minuta* essential oils on the three test bacteria species are shown in Table 7. In the case of *X. axonopodis* pv. *manihotis*, there was no visible bacterial growth on tubes 1-5 and 13. The lowest concentration of the essential oil at which no visible bacterial growth occurred in the MHB tubes was 48mg/ml, which was thus the minimum inhibitory concentration of *T. minuta* essential oil against *X. axonopodis* pv. *phaseoli.* Following subculturing of aliquots of the content from each tube onto Mueller-Hinton agar plates and incubation, growth occurred on plate 4-11 while no growth occurred on plates 1-3 and 13. Thus the lowest concentration of the essential oil at which no growth occurred on MHA plate after subculturing was 190mg/ml which was taken as the minimum bactericidal concentration of the oil against *X. axonopodis* pv. *manihotis.* The essential oils had MICs values of 24mg/ml in both *X. axonopodis* pv. *phaseoli* and *P. savastanoi* pv. *phaseolicola*, while a MBC value of 95mg/ml was recorded for the two fungi. Overall, the MICs value for the test bacteria ranged from 24 to 48mg/ml while the MBCs were in the range of 95-190mg/ml.

Table 7: Minimum inhibitory concentrations and minimum bactericidal concentrations of essential oils of *Tagetes minuta* on three plant pathogenic bacteria

Tube/Plate No.	EOs concentration (mg/ml)*10^2	Growth of bacteria in MHB tubes			Growth of bacteria in MHA plates		
		XAP	XAM	PSP	XAP	XAM	PSP
1	Crude EOs	-	-	-	-	-	-
2	3.8	-	-	-	-	-	-
3	1.9	-	-	-	-	-	-
4	0.95	-	-	-	-	+	-
5	0.48	-	-	-	+	+	+
6	0.24	-	+	-	+	+	+
7	0.12	+	+	+	+	+	+
8	0.06	+	+	+	+	+	+
9	0.03	+	+	+	+	+	+
10	0.015	+	+	+	+	+	+
11	- ve control	+	+	+	+	+	+
13	+ve control	-	-	-	-	-	-

[+] growth; [-] No growth of the bacteria; XAP - *X. axonopodis* pv. *phaseoli*, XAM - *X. axonopodis* pv. *manihotis* and PSP - *P. savastanoi* pv. *phaseolicola*
MHB - Muller-Hinton Broth
MHA - Muller-Hinton Agar
Tube 1 - Bacterial inoculum and undiluted crude essential oils
Tube 2 to 10 - MHB, bacterial inocula and essential oils of different concentrations
Tube 11 - Bacterial inoculum and MHB (-ve control)
Tube 13 - Bacterial inoculum, MHB and the standard bactericide (+ve control)

4.10 Chemical composition of *Tagetes minuta* essential oil

The chemical composition of *Tagetes minuta* essential oil was determined by gas chromatograph-mass spectrometry. The GC-MS analyses identified 20 compounds corresponding to 96% of the total oil. Table 8 shows the compounds identified from the essential oils of *T. minuta* along with the retention indices and concentration percentage. The essential oil comprised of hydrocarbons mainly terpenes; a mixture of monoterpenes and sesquiterpenes 70% and 30%, respectively. The most abundant monoterpenes which were also the most abundant components in the oil were (E)-Tagetone and dihydrotagetone accounting for 11.8 and 10.7% of the total oil, respectively. The least abundant monoterpenes were α-phellandrene and (Z)-β- ocimene each representing 3.6% of the total essential oil composition. On the other hand, sesquiterpenes concentration ranged from about 3.4 - 3.5% and mainly comprised of (E)-caryophyllene (3.5%), Elixene (3.5%), Bicyclogermacrene (3.5%), α-Humulene (3.5%), Silphiperfol-6-ene (3.4%) and one unknown sesquiterpene compound (3.5%). In summary, monoterpene hydrocarbons (30%), oxygenated monoterpenes (25%) and 3 unknown monoterpenes (15%) occurred in relatively high concentrations as compared to sesquiterpene hydrocarbons (25%) and non-oxygenated sesquiterpenes and one unknown sesquiterpenes (1%).

Table 8: The GC-MS identified constituents in the essential oil of *Tagetes minuta* sampled from Maseno, Kisumu County, Kenya

No[a]	Rt (min)	Compound Name	Molecular formula	M+g/mol	Retention index	Base peak	Major peaks	Concentration (%)±SE
1	10.6	Sabinene	$C_{10}H_{16}$	136.2	956	93	136, 121, 91, 79, 77, 69	3.8±0.12
2	11.2	α-Phellandrene	$C_{10}H_{16}$	136.2	983	93	136, 121, 94, 91, 80, 77	3.6±0.06
3	11.7	Limonene	$C_{10}H_{16}$	136.2	1005	68	136, 121, 107, 93, 79, 53	5.3±0.02
4	11.8	(Z)-β- Ocimene	$C_{10}H_{16}$	136.2	1015	93	121, 105, 79, 67, 53, 41	7.0±0.03
5	12.0	(E)-β- Ocimene	$C_{10}H_{16}$	136.2	1027	93	136, 121, 105, 79, 67, 53	3.6±0.01
6	12.1	Dihydrotagetone	$C_{10}H_{18}O$	154.2	1033	85	154, 139, 119, 97, 69, 57, 41	10.7±0.25
7	12.8	Unknown	-	-	1073	71	136, 121, 105, 93, 79, 67	5.4±0.01
8	13.4	Allo-Ocimene	$C_{10}H_{16}$	136.2	1110	121	136, 121, 105, 93, 79, 67	8.8±0.01
9	13.5	Unknown	-	-	1116	91	134, 119, 105, 91, 67, 55	3.9±0.01
10	13.6	Unknown	-	-	1122	43	139, 121, 111, 93, 81, 69	3.7±0.01
11	13.7	(Z)-Tagetone	$C_{10}H_{16}O$	152.2	1126	95	152, 134, 109, 93, 67, 43	5.0±0.06
12	13.8	(E)-Tagetone	$C_{10}H_{16}O$	152.2	1134	95	152, 134, 109, 93, 67, 43	11.8±0.02
13	15.0	(Z)- Ocimenone	$C_{10}H_{14}O$	150.2	1205	93	135, 121, 79, 69, 41	3.8±0.01
14	15.1	(E)- Ocimenone	$C_{10}H_{14}O$	150.2	1213	69	137, 119, 109, 91, 41	3.9±0.01
15	15.6	Unknown	-	-	1247	97	126, 83, 70, 55, 43	3.5±0.04
16	16.6	Elixene	$C_{15}H_{24}$	204.35	1310	121	204, 189, 162, 136, 107, 93	3.5±0.02
17	16.7	Silphiperfol-6-ene	$C_{15}H_{24}$	204.35	1319	133	107, 93, 79, 55, 41	3.4±0.01
18	17.7	(E)-Caryophyllene	$C_{15}H_{24}$	204.35	1393	93	133, 107, 105, 91, 79, 77	3.5±0.01
19	18.2	α-Humulene	$C_{15}H_{24}$	204.35	1400	93	147, 121, 107, 80 ,67	3.5±0.03
20	18.7	Bicyclogermacrene	$C_{15}H_{24}$	204.35	1401	93	204, 189, 161, 121, 107, 79	3.5±0.03

[a] = Peak numbers referring to Figure 12
SE = Standard error of the mean
M+g/mol = Molecular weight

Figure 12 shows the representative total ion chromatogram of *Tagetes minuta* essential oil in which separation of the twenty identified compounds was obtained. Each of the peaks represents the signal created when a particular component in the injected oil eluted from the gas chromatograph column to the detector.

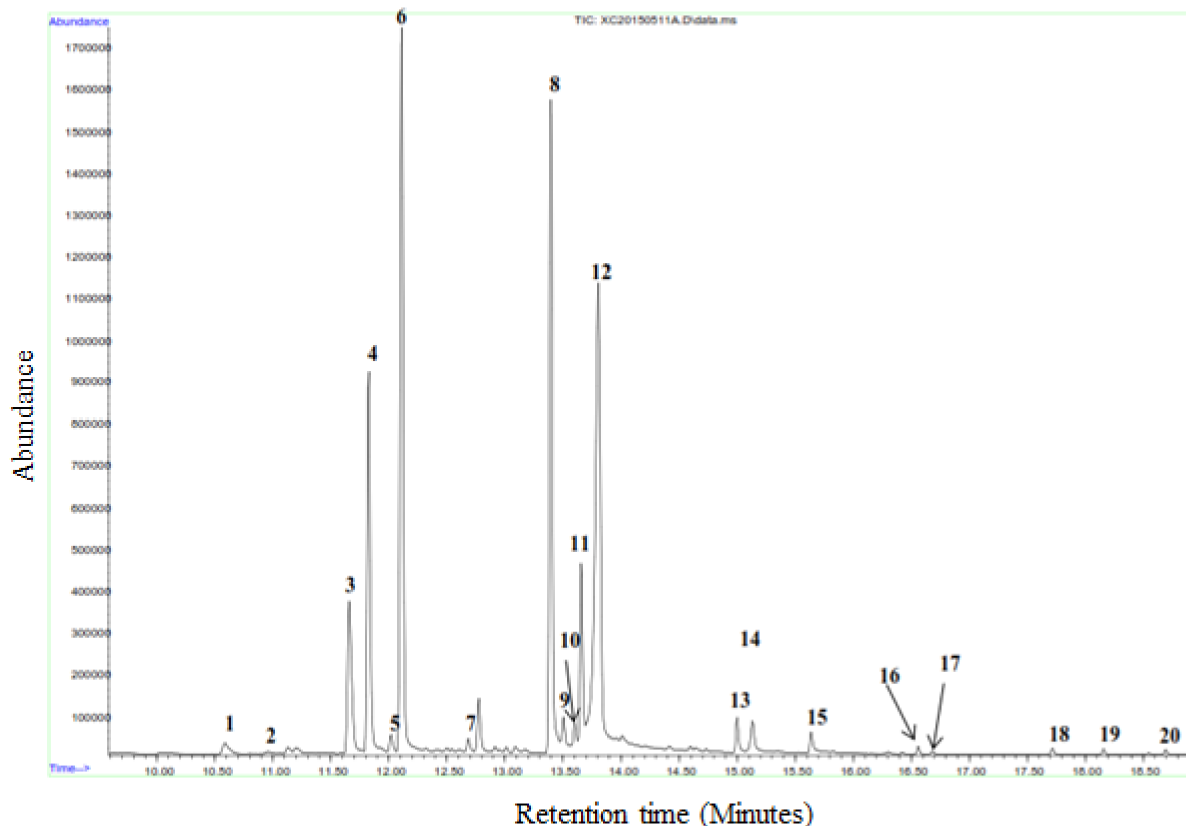

Figure 12: Representative total ion chromatogram of *Tagetes minuta* essential oil

The compound **(12)** occurring at a retention time of 13.8 min was the most abundant constituent in the essential oil with a concentration of 11.8% of the total oil. Compound (**12)** was identified as (E)-Tagetone also known as Trans-tagetone or by the International Union of Pure and Applied Chemistry (IUPAC) name of (5E)-2,6-dimethylocta-5,7-dien-4-one. (E)-Tagetone is classified as a monoterpene (a category of terpenes that consist of two isoprene units). Monoterpenes may be linear (acyclic), or may contain rings. (E)-tagetone is a linear monoterpene with a molecular formula $C_{10}H_{16}O$ and molecular weight of 152.20 g/mol. Another linear monoterpene identified in the essential oils was compound **(11)**, which was identified as identified as (Z)-Tagetone and which is an isomeric form of (E)-tagetone. (Z)-Tagetone also known as cis-tagetone and by the IUPAC name of (5Z)-2,6-dimethylocta-5,7-

dien-4-one has the same molecular formula and molecular weight as (E)-tagetone i.e. $C_{10}H_{16}O$ and 152.20 g/mol, respectively. (Z)-tagetone occurred at a retention time of 13.7 min with a relative abundance of 5.0% of the total essential oil.

Compound **(6)** occurring at retention time of 12.1 min was the second most abundant component identified in the oil constituting 10.7% of the total essential oil. This compound was identified as Dihydrotagetone known by the IUPAC name of 2,6-dimethyloct-7-en-4-one. Like (E) - and (Z)-tagetone, dihydrotagetone is an acyclic oxygenated monoterpene with a molecular formula of $C_{10}H_{18}O$ and a molecular weight of 154.20 g/mol. Allo-Ocimene also known as Allocymene and by the IUPAC name as (4E,6E)-2,6-dimethylocta-2,4,6-triene was another monoterpene identified as compound (**8**). Allo-Ocimene with a molecular formula of $C_{10}H_{16}$ and a molecular weight of 136.20 g/mol occurred at a retention time of 13.4 min, constituting 8.8% of the total oil. The major differences between allo-ocimene and the three monoterpenes already described i.e. (E)-, (Z)-tagetone and dihydrotagetone is that, while the three belong to a group of monoterpenes known as oxygenated monoterpenes (OT), allo-ocimene is classified as a hydrocarbon monoterpene (HT) since it lack an oxygen atom.

The compound **(4)**, occurring at a retention time of 11.8 min and constituting 7.0% of the total oil was identified as (Z)-β- Ocimene, an acyclic monoterpene. Since (Z)-β- ocimene belong to the same group of isomeric hydrocarbons with allo-ocimene i.e. Ocimenes, both have identical molecular formulae and molecular weights i.e. $C_{10}H_{16}$ and 136.20 g/mol, respectively. In addition to allo-ocimene and (Z)-β-ocimene, another ocimene identified in the essential oil was (E)-β-ocimene **(5)** which occurred at a retention time of 12.0 min and constituted approximately 3.6% of the essential oil. (E)-Ocimenone was identified as compound **(14)**, an oxygenated monoterpene also known as (E)-tagetenone, trans-ocimenone or trans-tagetenone and by the IUPAC name (5E)-2,6-dimethylocta-2,5,7-trien-4-one. (E)-ocimenone occurred at a retention time of 15.1 min and constituted 3.9% of the total essential oil. In addition to (E)-ocimenone, another Ocimenone i.e. (Z)-ocimenone **(13)** also known as (Z)-tagetenone, cis-tagetenone and by the IUPAC name (5Z)-2,6-dimethylocta-2,5,7-trien-4-one was identified in the oil. (Z)-ocimenone occurred at a retention time of 15.0 min and constituted about 3.8% of the oil. Both (E)-ocimenone and (Z)-ocimenone are acyclic oxygenated monoterpenes as shown by their respective chemical structures. Being isomers,

their molecular formulae and weights are the same i.e. $C_{10}H_{14}O$ and 150.20 g/mol, respectively.

Compound (**1**) occurring at retention time of 10.6 min was identified as Sabinene, a liquid monoterpene hydrocarbon with a molecular formula $C_{10}H_{16}$ and a molecular weight of 136.20 g/mol. Sabinene constituted 3.8% of the total oil. Sabinene alike all the monoterpenes described so far is a bicyclic unsaturated monoterpene. Another cyclic monoterpene identified in *T. minuta* essential oils was Limonene **(3)**. Limonene whose IUPAC name is 1-methyl-4-prop-1-en-2-ylcyclohexene occurred at a retention time of 11.7 min. It has a molecular formula of $C_{10}H_{16}$ and a molecular weight of 136.20 g/mol. Limonene takes its name form lemon, because limonene, (+/-) - a racemic mixture of limonene, which is a major component of the oil extracted from lemon and the rinds of other citrus fruits too contains considerable amounts of this compound. α-Phellandrene **(2)** is another cyclic monoterpene that was found in the essential oils. Alpha-phellandrene whose molecular formula and molecular weight are identical to those of limonene i.e. $C_{10}H_{16}$ and 136.20 g/mol, respectively gets its name from *Eucalyptus phellandra* (now known as *Eucalyptus radiate*) from which it can be isolated. This compound occurred at a retention time of 11.2 min and constituted 3.6% of the essential oil. The chemical structures of the monoterpenes identified n *T. minuta* essential oils are shown in Figure 13.

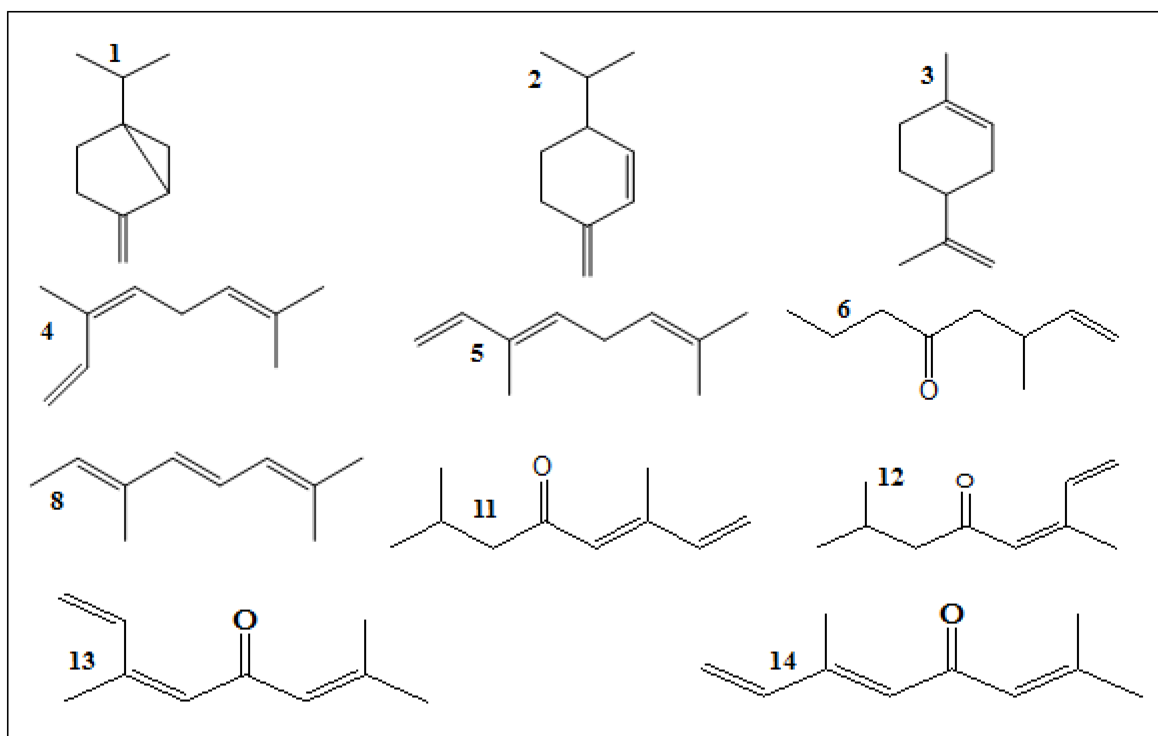

Figure 13: Chemical structures of monoterpenes identified in *Tagetes minuta* essential oils. (1) Sabinene, (2) α-Phellandrene, (3) Limonene, (4) (Z)-β-Ocimene, (5) (E)-β-Ocimene, (6) Dihydrotagetone, (8) Allo-Ocimene, (11) (Z)-Tagetone, (12) (E)-Tagetone, (13) (Z)-ocimenone (13) and (14) (E)-Ocimenone

In addition to monoterpenes, GC-MS analyses of *Tagetes minuta* essential oils revealed the presence of a number of sesquiterpenes which accounted for 30% of all the identified components. The concentration of the sesquiterpenes in the oil was in the range of 3.4-3.5%. Sesquiterpenes are a group of terpenes that consist of three isoprene units i.e. fifteen carbons and twenty-four hydrogen atoms per molecule. There are thousand kinds of sesquiterpenes which like monoterpenes may either contain rings or may be acyclic.

The compound **(16)** occurring at a retention time of 16.6 min and constituting about 3.5% of the total essential oil was identified as Elixene. Elixene also known by the IUPAC name 1-ethenyl-1-methyl-4-propan-2-ylidene-2-prop-1-en-2-ylcyclohexane is a cyclic sesquiterpene with a molecular formula of $C_{15}H_{24}$ and a molecular weight of 204.35 g/mol. Another sesquiterpene identified in the essential oils was compound **(17)** which occurred at a retention time of 16.7 min and accounted 3.4% of the oil. This compound was identified as Silphiperfol-6-ene. Silphiperfol-6-ene has a molecular formula and molecular weight that are identical to those of Elixene i.e. $C_{15}H_{24}$ and 204.35 g/mol, respectively. (E)-caryophyllene

was another sesquiterpene identified in the essential oil and represented by peak **(18)**. (E)-caryophyllene is a natural bicyclic sesquiterpene and occurred at a retention time 17.7 min and like elixene and silphiperfol-6-ene, has a molecular formula of $C_{15}H_{24}$ and a molecular weight of 204.35 g/mol. It accounted for 3.5% of the total essential oil.

Compound **(19)** was identified as α-Humulene, a sesquiterpene that is also known as Humulene and formerly as α-caryophyllene and whose IUPAC name is (1E,4E,8E)-2,6,6,9-tetramethylcycloundeca-1,4,8-triene. This compound occurred at a retention time of 18.2 and accounted for 3.5% of the oil. α-humulene is a naturally occurring monocyclic sesquiterpene with 11-membered ring and consisting of 3 isoprene units. Compound **(20)** was the last component to elute from the column and hence had the longest retention time of 18.7 min and constituted about 3.5% of the total oil. This compound was identified as a Bicyclogermacrene, a strained bicyclic sesquiterpene found in essential oils of several plants. The chemical structures of the sesquiterpenes identified in *Tagetes minuta* essential oils are shown in Figure 14. While a summary of the fragmentation pattern of all the terpenes identified in *Tagetes minuta* essential and major fragmentation ion and masses is shown in Appendix E

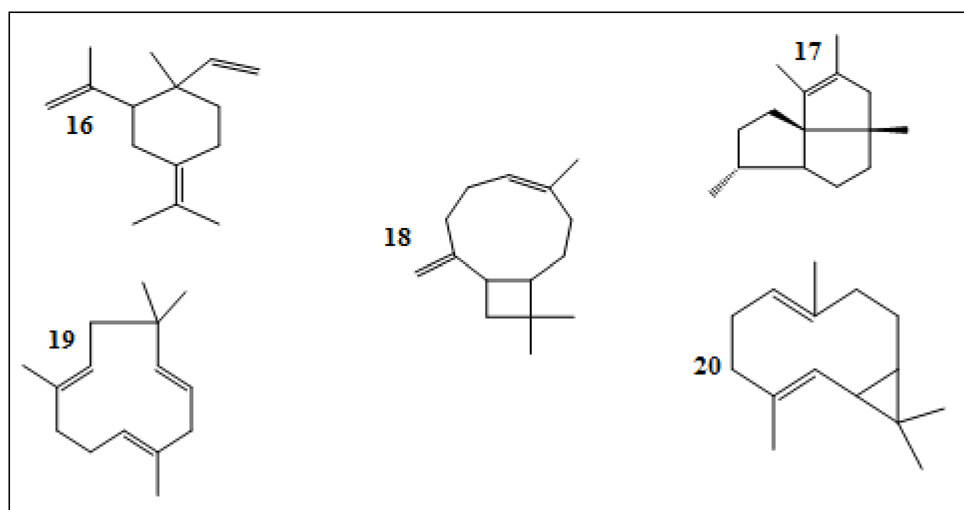

Figure 14: Chemical structures of sesquiterpenes identified in *Tagetes minuta* essential oils. (16) Elixene, (17) Silphiperfol-6-ene, (18) (E)-Caryophyllene, (19) α-Humulene and (20) Bicyclogermacrene

CHAPTER FIVE: DISCUSSION

This study aimed at extraction and evaluation of yields of the essential oils of *Tagetes minuta*, assessment of the antimicrobial activity of the essential oils against selected phytopathogens and characterization of the chemical composition of the essential oils. This therefore forms the basis of the discussion in this section.

5.1 Percentage yield of essential oils of *Tagetes minuta*

The mean percent yield of essential oils from fresh aerial parts (leaves, stems and flowers) of *Tagetes minuta* in the current study was 0.059% w/w. The steam distillation method had a relative standard deviation (RSD) of 1.01%. According to Caburian and Osi (2010), such a RSD value (specification: precision = maximum of 2% RSD) indicated that the steam distillation method was precise and with minimal wastages. The EOs percent yield obtained in the current study was higher than that obtained in previous studies from the same plant species sampled from other parts of the country but using different extraction methods. For instance, Wanzala (2009) and Wanzala and Ogoma (2013) obtained essential oil yields of 0.00029% w/w from fresh aerial parts of *T. minuta* plant materials obtained from Bungoma County, western Kenya. In another study, Makang'a (2012) obtained essential oil yields of 0.045, 0.039, and 0.035% w/w from *T. minuta* plant materials obtained from three regions in different agro-ecological zones in Kenya namely: Kasarani (Nairobi County), Bungoma (Bungoma County) and Bondo (Siaya County), respectively. However, much higher yields of *T. minuta* EOs than those obtained in the current study have been reported in literature. For instance, Karimian *et al*. (2014) obtained yields of 1% w/w from leaves of *T. minuta* plants grown in greenhouse conditions in Iran, while Meshkatalsadat *et al*. (2010) recorded yields of 1.2% w/w from *T. minuta* plants grown on experimental farms in the same country. The reported variations in the yields of essential oils of *T. minuta* and other botanically identical plant species such as *Salvia officinalis* L., and *Eucalyptus globulus* (Lakušić *et al*., 2013; Subramanian *et al*., 2012) could be attributed to various interactions between the genetics, ontogenesis and physiological state of the plant with the environment in addition to numerous abiotic factors that are present in the external environment of the growing plants (Lakušić *et al*., 2013; Wanzala and Ogoma, 2013; Lee and Phebe, 2016).

While the current study employed steam distillation to extract essential oils from freshly collected plant materials, the studies by Wanzala (2009), Wanzala and Ogoma (2013) and Makang'a (2012) employed hydrodistillation method to extract the oils from plant materials that had been left overnight in a ventilated room. Numerous studies have also reported variations in essential oil yields based on whether fresh or dried plant materials are used (Okoh *et al*., 2008; Sefidkon and Fathi, 2012). Thus, differences in the processing procedures of the plant materials before extraction of EOs among other factors may account for the variation in the yields of essential oils observed in the current study when compared to those recorded by Wanzala (2009), Wanzala and Ogoma (2013) and Makang'a (2012). Moreover, the variation in the extraction methods employed to isolate the essential oils could explain the differences in the yields of essential oil observed. While the three aforementioned studies employed hydrodistillation method, steam distillation method was used in the current study. Compared to hydrodistillation method, steam distillation method has been found to produce higher essential oils yields (Boutekedjiret *et al*., 2003; Tandon, 2008). In the steam distillation method, plant materials are supported on a perforated grid below which water is boiled. Therefore, direct contact of plant materials and the hot tank bottom is avoided and this reduces the chances of decomposition of thermally unstable compounds in the EOs (Tandon, 2008).

The difference in the yields of essential oils *T. minuta* has been reported in a number of studies based on the extraction methods and/or techniques used, in addition to variation in extraction conditions of temperature and pressure. Babu and Kaul (2007) analyzed the differences in both quantitative and qualitative characteristics of essential oils of *T. minuta* distilled in a vacuum and by conventional distillation technique at normal temperature and pressure (NTP). The results from the study revealed that higher yields of essential oils of *T. minuta* were obtained using the conventional distillation technique at NTP than in vacuum distillation. In addition to processing procedure of plant materials and method of extraction employed, yields of essential oils *T. minuta* have been found to vary considerably based on other factors such as the parts of the plant from, which the essential oils are extracted, harvesting season and plant growth stage (Senatore *et al*., 2004; Chamorro *et al*., 2008). In regard to parts of the plant from which the oils are extracted, Saha *et al*. (2012) found the

yields of *T. minuta* EOs extracted from the leaves and flowers to fall within the ranges of 0.3-0.4% and 0.2-0.3%, respectively. Moradalizadeh *et al*. (2013) on the other hand obtained yields of 0.9, 0.5 and 0.7 % (w/w) from leaves, seeds and flowers of *T. minuta*, respectively.

In the current study, essential oils were extracted from *T. minuta* plants collected during the flowering stage. The growth stages at which plant materials are collected for extraction of essential oil have been shown to affect the yields. For instance, Singh *et al*. (2006) evaluated the quality and yields of essential oils from cultivated *T. minuta* at different harvest indexes. The study revealed that maximum yields of essential oils were obtained from plants collected at post flowering stage (seed setting stage). The advantage in yields of essential oil at the post flowering stage was found to be 130%, 34.5% and 36.5% yields enhancement over pre-flowering, 50% and 100% flowering stages, respectively. Further, Singh *et al*. (2006) evaluated the maximum yields of essential oils that different plant parts could give. The study revealed that flowers and leaves contained maximum essential oil of 2.14% and 1.89% (dry weight basis), respectively with the relative contribution of leaf, flower and stem to essential oil production being 40.7%, 37.8% and 21.5%, respectively. The findings were in agreement with those of Thappa *et al*. (1993) who reported the yields of essential oils of 1.14%, 1.00 and 1.25% at full bloom, full bloom and immature fruit and fruit maturation stages, of *T. minuta*, respectively.

Differences in the yields of essential oils of *T. minuta* have also been reported on the basis of edaphic factors such as soil types, nutrient status in addition to other planting parameters such as plant spacing in the case of cultivated *T. minuta* (Graven *et al*., 1991; Singh *et al*., 2008). The levels of important plant nutrients such as nitrogen (Singh *et al*., 2008; Omidbaigi *et al.* 2008), phosphorous (Graven *et al*., 1991; Negahban *et al*., 2014) and Sulphur (Graven *et al*., 1991) have all been found to have an influence on the yields of essential oils of *T. minuta.* The higher yields obtained in the current study could therefore be attributed to the extraction method used (steam distillation), extraction of the essential oils from freshly collected rather than dried plant material and the harvesting of the plant materials for EOs extraction at the flowering stage.

5.2. Antimicrobial activity of essential oils of *Tagetes minuta*

Tagetes minuta essential oils were found to have potent antifungal activity against all the test fungal species, namely: *Fusarium oxysporum, Fusarium solani, Aspergillus flavus*, *Aspergillus parasiticus,* and *Aspergillus niger*. The antifungal activities of crude essential oils varied from one test species to another with four tested fungal species - *F. oxysporum*, *A. flavus*, *A. parasiticus* and *A. niger* - falling within the category of extremely sensitive (diameters of the inhibition zone larger than 20mm) while *F. solani* was ranked as very sensitive (diameters of the inhibition zone between 15 and 19mm) after five days of incubation. The study's findings were thus in agreement with the reported promising antifungal activity of essential oils, aqueous and organic extracts of *T. minuta* against a wide range of economically important phytopathogens (Muyima *et al*., 2004; Shirazi *et al*., 2014; Uzabakiriho *et al*., 2015).

The highest activity of crude essential oils was observed in *F. oxysporum* and *A. niger* both of which had mean inhibition zones of 28.67mm, while the lowest activity was recorded in *F. solani* with a mean inhibition zone of 19.83mm after five days of incubation. The findings on antifungal activity of *T. minuta* essential oils in the current study were in agreement with the results by Saha *et al*. (2012) who studied the antifungal activity of essential oils from leaves and flowers, and thiopene rich extracts from roots and shoots of *T. minuta* against eight plant pathogenic fungi namely; *Rhizoctonia solani*, *Fusarium solani*, *Sclerotinia sclerotiorum*, *Fusarium oxysporum pisi*, *Fusarium oxysporum lentis*, *Sclerotium rolfsii, Pyricularia grisea* and *Alternaria solani.* The study revealed that leaf EOs and root extract had the highest inhibitory activity against all the studied plant pathogens. Essential oils from leaves showed hyphal growth inhibition of 34.1 to 85.6% while root extracts showed inhibitions of 21.6 to 77.8% after 6 days against the studied phytopathogens (Saha *et al*., 2012).

The study showed that the activity of crude essential oils was significantly higher ($p \leq 0.05$) on the 5th day of incubation in comparison with the activities recorded after ten days of incubation in four out of the five studied fungi. The reduction in the antifungal activity of the EOs within the two incubation periods i.e. 5th and 10th day could be attributed to the reduction in the oil's strength due to its high volatility and hence a reduction in the content of some or all of the active principles among other factors (Sumalan *et al*., 2013). The highest

inhibition of fungal growth was observed after 5 days of incubation and decrease significantly thereafter for all the fungi. These findings concur with a number of studies that have reported the optimum antifungal activity of some essential oils to fall within the 5th and 7th day of incubation (Clara 2013; Sumalan *et al*., 2013). Similar observations were made by Císarová *et al*. (2014) who studied the activity of EOs of *Thymus vulgaris* L., *Syzygium aromaticum* (L.) Merrill & Perry, *Ocimum basilicum* L., *Jasminum officinale* L., and *Rosmarinus officinalis* L. against common fungi causing spoilage of bakery products. The study revealed that the highest activity of the five essential oils were recorded on the 7th day, with the activity of most oils reducing or ceasing completely between the 14th and 21st day of incubation.

The antifungal bioassay with the crude essential oils revealed that in addition to growth inhibitory activity as was revealed by inhibition zones of different sizes, the essential oils caused a delay in the growth in *A. niger*, *A. parasiticus* and *A. flavus*. The growth rate of the three fungi after treatment with the EOs was lower when compared to their negative treatments. These observations may be attributed to the ability of the essential oils to change the general growth and morphogenesis of fungi as previously reported by De Billerbeck *et al*. (2001) and Sharma and Tripathi (2008). In the two studies, microscopic analysis revealed that the essential oils of *Cymbopogon nardus* (*L.*) Rendle and *Citrus sinensis* (L.) Osbeck produced some deleterious ultrastructural modifications in *A. niger* including loss of cytoplasm in fungal hyphae, progressive thinning of hyphal diameter and walls, budding and flattening of hyphal tip, plasma membrane disruption and mitochondrial structure disorganization (De Billerbeck *et al*., 2001; Sharma and Tripathi, 2008). The modifications in cytological structures were attributed to the interference of the essential oils with the enzymes responsible for wall synthesis (De Billerbeck *et al*., 2001). Considerable changes in mycelial growth and sporulation were also reported by Silva *et al*. (2012) who studied the effects of essential oils of *Foeniculum vulgare* Mill, *Zingiber officinale* Roscoe, *Mentha piperita* L., and *Thymus vulgaris* L., on *A. flavus* and *A. parasiticus.* The authors noted significant inhibitory effects on mycelial growth of *A. parasiticus* by the four EOs with thyme EO showing the best inhibitory effect on mycelial growth and sporulation of *A. parasiticus* and *A. flavus*.

In the current study, it was established that for *A. flavus* and *A. parasiticus*, growth inhibition occurred even in regions of the Petri plates that were not in direct contact with the oil-impregnated paper discs. This was an indication that the essential oils were able to inhibit growth of the two fungi in regions far removed from the primary essential oil application cites. The ability of the essential oils to cause growth inhibition in fungi without any direct contact is attributed to the vapour-phase antifungal activities of the essential oils. There is evidence in literature on the fungistatic and fungicidal activity of volatile vapours of essential oils through inhibition of mycelial growth and sporulation (Chee and Lee, 2004; Aguiar *et al*., 2014). Chee and Lee (2007) reported that volatile vapours of clove essential oils exhibited potent fungistatic activity whereas direct application of essential oil showed fungicidal activity against dermatophytic fungi *Epidermophyton floccosum*, *Microsporum audouinii*, *Trichophyton mentagrophytes*, and *Trichophyton rubrum.* The activity of the essential oil against the aforementioned dermatophytes was shown to occur through inhibition of mycelial growth and spore germination.

The minimum inhibitory concentrations (MICs) of the essential oils of *T. minuta* on the studied fungi ranged between 24 – 95 mg/ml. However, the MICs values obtained through the tube dilution method were different from the MICs estimates obtained using the disc diffusion method i.e. 6 – 24 mg/ml. The variation in the results from the two methods is indicative of the difficulty of comparing antimicrobial susceptibility testing results that have been conducted using different methodologies, especially in regard to the minimal inhibitory concentrations (Hili *et al*., 1997; Suhr and Nielsen, 2003). In the current study, differences in the solubility, diffusion and evaporation rates of the essential oils in agar and broth media could be among the paramount factors that contributed to the observed differences between the two methods (Saidana *et al*., 2008). The minimum fungicidal concentrations (MFCs) were in the range of 24 -190 mg/ml. *Aspergillus niger* and *A. parasiticus* had MICs values of 24 and 48 mg/ml, respectively. For these two fungi therefore, the lowest concentration of EOs at which no visible growth was observed (judged by the naked eye) in the broth tube was also the lowest concentration at which the EOs produced biocidal effects on the fungi.

Tagetes minuta essential oils had strong antibacterial activities against the three test phytopathogenic bacteria namely; *Pseudomonas savastanoi* pv. *phaseolicola, Xanthomonas*

axonopodis pv. *phaseoli* and *Xanthomonas axonopodis* pv. *manihotis.* The activity of crude essential oils against the three bacteria was within the category of extremely sensitive (diameters of the inhibition zone larger than 20 mm) after 24 and 48 hours of incubation. The highest antibacterial activity of crude essential oil was observed in *P. savastanoi* pv. *phaseolicola* with mean inhibition zone of 41.83 mm while in *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *manihotis,* the oils produced inhibition zones of 26.83 mm after 24 hours of incubation. Antibacterial activities of *T. minuta* EOs have been reported in literature with most studies conducted on human pathogenic bacteria (Muyima *et al.,* 2004; Shirazi *et al*., 2014). Studies on the antimicrobial activities of essential oils from *T. minuta* and other plant species have generally established that Gram-positive bacteria are more susceptible to the effects of EOs than Gram-negative bacteria (Muyima *et al.,* 2004; Trombetta *et al*., 2005). However, the three bacteria used in the current study were all Gram-negative hence a comparison of this nature is not feasible. The MICs of the EOs against the test bacteria were in the range of 24 - 48 mg/mL. The MIC values recorded in the current study were much higher than those reported in a number of studies. For instance, Shirazi *et al*. (2014) reported MICs of 150 ± 8, 165 ± 9, 67 ± 8, 75 ± 7, 135 ± 15, and 115 ± 8 μg/mL of *T. minuta* EOs against *Salmonella typhi*, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Aspergillus niger*, and *Candida albicans*, respectively. Da Cunha *et al*., (2016) obtained MICs of *T. minuta* EOs ranging from 1.28 – 51.4µg/mL against some *Cryptococcus* spp. isolates.

Variation in the antibacterial activities of *T. minuta* essential oils with respect to the two specified incubation periods i.e. 24 and 48 hours was statistically significant ($p \leq 0.05$), for the three bacteria. Enrich BM®, the standard bactericide exhibited the highest activity in *X. axonopodis* pv. *manihotis* for both 24 and 48 hours of incubation periods. This implies that, while *X. axonopodis* pv. *manihotis* was the least susceptible bacteria to the inhibitory action of the essential oils in comparison with the rest of the tested bacteria, it was the most susceptible to the standard bactericide within the two incubation regimes. The activity of the standard bactericide against *X. axonopodis* pv. *phaseoli* and *P. savastanoi* pv. *phaseolicola* was not significantly different within the two specified periods, while for *X. axonopodis* pv. *manihotis,* the activity of Enrich BM® was significantly higher after 48 hours of incubation in comparison with the activity recorded after 24 hours of incubation.

The study revealed a concentration-dependent inhibitory activity of EOs against the tested fungi and bacteria. In general, increase in the concentration of essential oils resulted in an increase in the diameters of the inhibition zone and thus, dose-dependent response was clear in most of the tested fungi and bacteria. It has previously been reported that most biological activities including antimicrobial activity of essential oils are dependent on their concentrations (Lodhia *et al*., 2009; Silva *et al*., 2012; Makang'a, 2012). Muyima *et al.* (2004) and Shirazi *et al*. (2014) reported concentration-dependent antibacterial and antifungal activity of *T. minuta* EOs against a wide range of bacterial and fungal species. The concentration-dependent antimicrobial activities of EOs observed were attributed to changes in concentration of the active principles as the overall EO concentration changed. This view is supported by a number of studies that have reported dose-dependent biological activities of various pure compounds isolated from essential oils (Tabanca *et al*., 2007). Thus, as the concentration of the EO increases, so does the active component present in the oil and hence an increase in the activity of the oil in this case represented by larger inhibition zones. A significant linear correlation ($p \leq 0.05$) between the essential oils concentration and inhibition of bacterial growth was observed in the three studied bacteria. The same applied to three out of five tested fungi, namely: *A. parasiticus, A. flavus* and *F. solani*. However, no significant correlation between the essential oil concentration and the inhibition of fungal growth was observed in *A. niger* and *F. oxysporum*.

The study nevertheless, found some exceptional instances where essential oils that were more concentrated produced smaller inhibition zones in comparison to the less concentrated/more diluted EOs. This could explain the lack of a linear correlation between the essential oil concentration and growth inhibition of *A. niger* and *F. oxysporum.* Instances where more concentrated EOs produce smaller inhibition zones have been reported in literature and are attributed to the fact that dilute EOs diffused more easily in the agar medium (i.e. aqueous environment) than the undiluted or less dilute EOs (Hood *et al.,* 2003; Muyima *et al*., 2004). Moreover, polymerization of the undiluted essential oils contributes to the above observation (Muyima *et al*., 2004). It can therefore be concluded that antimicrobial activity of essential oils is a function of concentration, solubility, diffusion and evaporation rates of the essential oils in the media among other factors (Gomez-Lopez *et al*., 2005; Lalitha, 2014). Furthermore, the levels of dilutions, common solvents used and solubilizing agents such as

dimethyl sulphoxide, ethanol and Tween 80 have significant effects on antimicrobial susceptibility testing results (Gomez-Lopez *et al*., 2005; Jović *et al*., 2012).

5.3 Chemical composition of the essential oils of *Tagetes minuta*

Gas chromatograph - mass spectrometry (GC-MS) analyses of the essential oils of *T. minuta* identified twenty compounds, sixteen of which were conclusively identified and named while three monoterpenes and one sesquiterpene were unknown. The identity of the subclasses of the four unknown compounds was based on their mass-spectral fragmentation patterns in addition to their retention indicies. Great variations have been reported in literature on the chemical profiles of essential oils of *Tagetes minuta* sampled from a wide range of geographical locations. Some studies have reported as few as six compounds (Moghaddam *et al*., 2007; Chamorro *et al*., 2008) while others have reported over a hundred compounds (Wanzala and Ogoma, 2013) in *T. minuta* essential oils. Variations in the chemical composition of *T. minuta* essential oils with respect to harvesting location (Senatore *et al*., 2004; Chamorro *et al*., 2008), the growth stage (Chalchat *et al.,* 1995; Moghaddam *et al*., 2007) the different parts of the plant used (Weaver *et al*., 1994; Chalchat *et al.,* 1995), climatic condition under which the plant is grown (Mohamed *et al*., 2002) and the different chemotypes (Gil *et al*., 2000) have all been reported. Despite reported differences in the actual number of compounds identified in *T. minuta* EOs in different studies, majority of the compounds that have been identified as common constituents are terpenes belonging to any of the four terpene subclasses namely; monoterpenes, sesquiterpenes, hemiterpenes and diterpenes (Makang'a, 2012; Wanzala and Ogoma, 2013), suggesting that the chemical profiles of *T. minuta* EOs from different regions could be chemically comparable despite the great variation in the actual number of components present.

The current study identified a mixture of monoterpenes (70%) and sesquiterpenes (30%) in the oil. The findings of the study were in agreement with earlier reports that have cited monoterpenes and sesquiterpenes as the major groups of terpenes that are present in *T. minuta* essential oils. Makang'a (2012) found the composition of *T. minuta* EOs from three regions in Kenya namely; Kasarani (Nairobi County), Bungoma (Bungoma County) and Bondo (Siaya County) to contain mostly monoterpenes and sesquiterpenes hydrocarbons in the ratios of 3:7, 2:8 and 4:6, respectively. Wanzala and Ogoma (2013) identified 119

compounds in *T. minuta* oil represented by Monoterpenes (47.9%), Sesquiterpenes (30.3%), Hemiterpenes (15.1%), Diterpenes (1.7%) and Unknown (5.0%). Similarly, Chisowa *et al*. (1998) and Moghaddam *et al*. (2007) reported the two subclasses of terpenes i.e. monoterpenes and sesquiterpenes to constitute the biggest percentage of *T. minuta* oils.

Among the compounds identified in EOs in the current study, (E)-tagetone was the most abundant constituent accounting for 11.8% of the total oil followed by dihydrotagetone and allo-ocimene with 10.7 and 8.8%, respectively. These findings were comparable with a number of earlier qualitative studies on the chemical composition of EOs of *T. minuta* that have identified dihydrotagetone, tagetones, ocimenes, limonene and ocimenones as the most abundant constituents of *T. minuta* essential oils. In a study by Garcia *et al*. (2012) for instance, four monoterpene constituents - limonene, β-ocimene, dihydrotagetone and tagetone - were found to represent more than 70% of *T. minuta* essential oil. Dihydrotagetone has been cited as one of the most abundant constituents of *T. minuta* oils from plants sampled from a wide range of geographical locations. In Kenya for instance, dihydrotagetone has been cited as a principal constituent of *T. minuta* essential oil (Wanzala, 2009; Makang'a 2012; Wanzala and Ogoma, 2013). Moreover, dihydrotagetone has been identified as the most abundant component in *T. minuta* EOs from Iran (Shirazi *et al*., 2014), Zambia (Chisowa *et al*., 1998) and the UK (Senatore *et al*., 2004) constituting 33.9, 30.0 and 34.3% of the total oil, respectively.

Ocimenes have been reported as some of the most commonly encountered monoterpenes in *T. minuta* EOs. Ocimenes are isomeric monoterpenes that occur naturally in several plants. They are thermally unstable compounds that are commonly used in the pharmaceutical and fine-chemicals industries because of their natural plant defense properties and pleasant odour (Chamorro *et al*., 2014). Allo-ocimene was among the most abundant compound isolated from *T. minuta* constituting 8.8% of the total essential oils. The compound is a clear colorless to slightly yellowish liquid which is found in abundance within a variety of plants and fruits and is used in the manufacture of flavor and fragrance agents, in perfumery industry, as a diluting agent for varnishes and dyes and as a component for terpene polymers (Makang'a 2012). This compound has been found in *T. minuta* essential oils from different countries

such as Kenya (Onyambu *et al*., 2015), Iran (Shirazi *et al*., 2014), India (Upadhyaya *et al*., 2010) and Argentina (Vázquez *et al*., 2011).

In addition to Allo-ocimene, two other ocimenes were identified in the essential oils i.e. (E)-β-ocimene and (Z)-β-ocimene. (E)-β-ocimene is a colorless liquid and has been employed as a botanical insecticide and a solvent for cleaning purposes (Odalo *et al*., 2005). It has been identified as a component of *T. minuta* essential oil in a number of studies (Senatore *et al*., 2004; Ali *et al*., 2014). (Z)-β-ocimene has been cited as a component in *T. minuta* oil sampled from Zambia (Chisowa *et al*., 1998), Yemen (Ali *et al*., 2014) and India (Thappa *et al*., 1993). Two ocimenones i.e. (E) - and (Z)-ocimenone were also were identified in the oil. Ocimenones are oxygenated monoterpene and together with tagetones are important odour components of marigold flowers (Sell, 2003). Ocimenones have been cited as some of the most common constituents of *T. minuta* EOs. Ali *et al*. (2014) found (E)-ocimenone to be the most abundant component of *T. minuta* essential oil constituting 34.8% of the total oil. Both (E) - and (Z)-ocimenone have also been identified in EOs of *T. minuta* sampled from Kenya (Wanzala and Ogoma 2013), Egypt (Mahmoud, 2013), Iran (Moghaddam *et al*., 2007; Shirazi *et al*., 2014), Rwanda and France (Chalchat *et al*., 1995), Yemen (Ali *et al*., 2014) and India (Singh *et al*., 1992).

Sabinene, whose concentration in the current study was 3.8% is a natural bicyclic monoterpene that accumulates limitedly in natural organisms and has been identified both as a major (Aligiannis *et al*., 2001) and minor constituent (Ali *et al*., 2014; Shirazi *et al*., 2014) in EOs of several plants. Sabinene has been reported in EOs of *T. minuta* collected from Nairobi, western and Nyanza regions in Kenya at concentrations of 0.74, 0.38 and 0.38%, respectively (Makang'a, 2012). Wanzala and Ogoma (2013) likewise found trace amount of Sabinene as one of the components of *T. minuta* essential oils from plants sampled in Bungoma County, Western Kenya. Sabinene has also been reported in *T. minuta* EOs from Yemen (Ali *et al*., 2014), Iran (Shirazi *et al*., 2014), the UK (Senatore *et al*., 2004) and South Africa (Muyima *et al*., 2004) where it accounted for 0.3, 0.4, 1.8 and 3.65% of the composition of the essential oils, respectively.

Other monoterpenes that were identified in the EOs that have reported in literature as common constituents of *T. minuta* essential EOs include α-Phellandrene and limonene. Phellandrenes are widely used in fragrances because of their pleasing aromas (Pirbalouti and Aghaee, 2011). Limonene whose concentration in the current study was 5.3% takes its name from lemon, because limonene (+/-), a racemic mixture of limonene, which is the predominant compound in essential oils extracted from lemon and other citrus fruits contains considerable amounts of this compound. It is this compound that contributes to the strong smell of citrus fruits (Rabinski, 2015). Limonene is a colourless liquid that is commonly used in chemical synthesis as a precursor to carvone, as a fragrance in perfumery, a renewables-based solvent in cleaning products, in food manufacturing and also in some medicines where it acts as a flavouring agent to mask the bitter taste of alkaloids (Vummaneni and Nagpal, 2012). Limonene has been cited as the most abundant component in *T. minuta* EOs (Meshkatalsadat *et al*., 2010) and among the principal constituents of the essential oil (Muyima *et al*., 2004; Ali *et al*., 2014; Karimian *et al*., 2014).

Besides monoterpenes, sesquiterpenes were identified in *T. minuta* essential oils and included bicyclogermacrene, (E)-caryophyllene, elixene, α-humulene, and silphiperfol-6-ene with a concentration range of 3.4 - 3.5%. Bicyclogermacrene has been identified in EOs of *T. minuta* sampled from a wide geographical location at varying concentrations such as in Yemen 0.5% (Ali *et al*., 2014), Kenya 1.8% (Makang'a, 2012), Iran 0.9% (Moradalizadeh *et al*., 2013), India 2.7% (Upadhyaya *et al.,* 2010) and Argentina 6.8% (Vázquez et al., 2011). α-Humulene whose concentration in the current study was 3.5% has also been cited as common sesquiterpene in *T. minuta* EOs. It has been isolated in EOs of *T. minuta* collected from Kenya (Makang'a, 2012; Onyambu *et al*., 2015), Yemen (Ali *et al*., 2014) and Iran (Shirazi *et al*., 2014). (E)-caryophyllene, a cyclic sesquiterpene that is a constituent of essential oils of many aromatic plants was another compound identified in the oil. (E)-caryophyllene has been identified in *T. minuta* essential oil from Yemen (Ali *et al*., 2014) and Iran (Shirazi *et al*., 2014; Karimian *et al*., 2014). Both alpha-humulene and (E)-caryophyllene have been shown to have pronounced anti-inflammatory properties and hence are considered as candidate compounds for development of drugs for the management and/or treatment of inflammatory diseases (Fernandes *et al*., 2007; Rogerio *et al*., 2009).

This is the first report of Silphiperfol-6-ene and Elixene in *T. minuta* essential oils. Nevertheless, Silphiperfol-6-ene compound has been isolated from essential oils of a number of other plants such as *Fuerstia africana* T.C.E. Fries (Onyambu *et al*., 2015), *Silphium integrifolium* Michx and *Silphium trifoliatum* L (Kowalski, 2008) and *Anemia tomentosa* var. *anthriscifolia* Schrad (Pinto *et al*., 2009). Similarly, Elixene, a sesquiterpene has been reported as a principal component of essential oils of *Schinus terebinthifolia* Raddi (Barbosa *et al.,* 2007; Cole *et al.,* 2014), *Eugenia platysema* O. Berg (Tenfen *et al*., 2015) and *Mentha aquatic* L (Andro *et al.,* 2013). The two compounds - Silphiperfol-6-ene and Elixene - therefore could represent a new addition to the list of the constituents identified in *T. minuta* essential oils.

Antimicrobial activities of essential oils are attributed mainly to the major components and to some extent the minor components present in the EOs (Burt, 2004). Furthermore, synergistic interactions between the major and minor essential oil components have been reported (Bassolé and Juliani 2012). It has been widely reported in literature that generally, essential oils that contain phenolic structures such as carvacrol, eugenol and thymol produce the strongest antimicrobial activity (Dorman and Deans, 2000; Lambert *et al*., 2001). In the current study however, no phenolic compounds were identified in the essential oils of *T. minuta*. Thus, the bioactivity exhibited by the essential oils against the test fungal and bacterial pathogens was attributed to the monoterpenes and sesquiterpenes present in the oils. Previous investigations have attributed antimicrobial activity of *T. minuta* essential oil to the presence of ketone fractions (Senatore *et al*., 2004). Consequently, the antimicrobial activity of the essential oils of *T. minuta* observed in the current study could be attributed to the levels of tagetones, dihydrotagetones and ocimenones which were among the most abundant monoterpenes identified in the EOs. This view is supported by a number of studies that have reported strong antimicrobial activity of *T. minuta* EOs containing the aforementioned compounds (Senatore *et al*., 2004; Muyima *et al.,* 2013; Shirazi *et al*., 2014).

CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Phytochemicals are recognised as some of the most promising compounds in the development of new biopesticides for the management of plant pathogens. Compared to synthetic pesticides, plant-derived antimicrobials have numerous advantages such as high degradability, multiple mechanisms of action, no residual effects, fewer incidences of development of resistance among the target pathogens and generally low mammalian toxicity.

Data generated from this study showed that the percentage yield of *T. minuta* essential oil was relatively higher compared to findings in previous studies carried out in Kenya. The higher yields of essential oil of *T. minuta* recorded in this study could be attributed to several factors, namely: extraction method employed (i.e. steam distillation), distillation of fresh rather than dried plant materials, and collection of the plant materials at the flowering stage. These factors have been found to contribute to higher yields of essential oils from *T. minuta* in previous studies. The higher yields of essential oils of *T. minuta* offer promising prospects for commercialization and sustainable exploitation of the EOs for the development of essential oil-based pesticides.

Overall, the study revealed promising antimicrobial activities of *T. minuta* essential oils against the test phytopathogens. Out of the five studied fungi, four were within the category of extremely sensitive (inhibition zone diameters ≥ 20mm) with one being ranked very sensitive (inhibition zone diameters between 15 and 19mm) when subjected to crude essential oils of *T. minuta*. On the other hand, all the three test bacterial pathogens were extremely sensitive (inhibition zone diameters ≥ 20mm) to the crude essential oils of *T. minuta.* Furthermore, useful information on a number of antimicrobial activity parameters such as minimum inhibitory concentrations and minimum bactericidal/fungicidal concentrations was generated. Such data would be useful in the design and formulation of effective doses for a biopesticide that is based on *T. minuta* essential oils.

Twenty compounds - a mixture of monoterpenes and sesquiterpenes – were identified from the essential oils of *T. minuta.* The major compounds identified in the EOs were: (E)-

Tagetone (11.8%), dihydrotagetone (10.7%), Allo-Ocimene (8.8%), (Z)-β- Ocimene (7.0%), Limonene (5.3%) and (Z)-Tagetone (5.0%). These compounds have been cited as common constituents of essential oils of *T. minuta* from previous studies.

One of the most significant findings of this study was the identification of two compounds - Elixene and Silphiperfol-6-ene - that had not been previously reported in essential oils of *T. minuta*. This is therefore the first reported occurrence of the two sesquiterpenes in the essential oils of *T. minuta*, an important addition to the database of the chemical profile of *T. minuta* essential oils.

The study therefore lays down significant groundwork for a more comprehensive study on the potential application of *Tagetes minuta* essential oils in the development of novel, affordable and eco-friendly biopesticides for management of economically important phytopathogens.

6.2 Recommendations

The following recommendations are made:

i. There is need for evaluation of the antimicrobial activity of *Tagetes minuta* essential oils against the tested phytopathogens under field conditions.
ii. There is need for further investigation and bioassay-guided isolation and characterization of pure active compounds from *Tagetes minuta* EOs that confer the observed antimicrobial activity.
iii. *Tagetes minuta* essential oils were extracted from plants sampled at flowering stage during the October - November season within Kisumu County. Further studies are recommended on the effect of seasons, plant drying procedures, extraction methods and plant growth stages on yields and chemical composition of essential oils.
iv. The activity of *Tagetes minuta* essential oils against other economically important human and crop pathogens should be evaluated to assess the potential application of the EOs in pharmaceutical, agriculture and food industries.
v. Four out of five studied fungi that were studied in the current work are mycotoxigenic. A study is therefore recommended on the effect of essential oils on levels of mycotoxin biosynthesis in addition to the modes of action of the essential oils against the studied fungi.

vi. The identity of the four unknown compounds, which were not found in the GC-MS library, should be determined using other techniques such as nuclear magnetic resonance (NMR).
vii. There is a need to conduct toxicity tests on the essential oils of *Tagetes minuta* in order to assess its safety.
viii. The shelf life of the EOs should be determined to assess their rate of degradation and persistence as biological pesticides.

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APPENDICES

Appendix A: Analysis of variance (ANOVA) on the inhibition of *Tagetes minuta* essential oils and Apron star® on five fungal pathogens after five and ten days of incubation

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	2	1.346	0.673	0.31	
Fungi	4	512.299	128.075	58.7	<0.001
Days	1	162.361	162.361	74.41	<0.001
Oil-Apron Star	1	9.048	9.048	4.15	0.049
Fungi-Days	4	53.596	13.399	6.14	<0.001
Fungi-Oil-Apron Star	4	181.826	45.456	20.83	<0.001
Days-Oil-Apron-Star	1	140.148	140.148	64.23	<0.001
Fungi-Days-Oil-Apron Star	4	29.743	7.436	3.41	0.018
Residual	38	82.914	2.182		
Total	59	1173.28			

Appendix B: Linear regression analysis of inhibition zone diameters against the concentration of *Tagetes minuta* essential oils for the five test fungi

A. flavus

Parameter	Estimate	s. e.	t(5)	t pr.
Constant	7.3	1.53	4.83	0.005
EO Concentration	0.037	0.009	4.1	0.009
Parameter	Lower 95%	Upper 95%		
Constant	3.465	11.34		
EO Concentration	0.014	0.061		

A. niger

Parameter	Estimate	s. e.	t(5)	t pr.
Constant	11.5	2.26	5.09	0.004
EO Concentration	0.028	0.013	2.08	0.092
Parameter	Lower 95%	Upper 95%		
Constant	5.687	17.31		
EO Concentration	-0.006	0.063		

A. parasiticus

Parameter	Estimate	s. e.	t(5)	t pr.
Constant	7.42	1.34	5.54	0.003
EO Concentration	0.04	0.008	5.84	0.002
Parameter	Lower 95%	Upper 95%		
Constant	3.976	10.86		
EO Concentration	0.02	0.06		

F. oxysporum

Parameter	Estimate	s. e.	t(5)	t pr.
Constant	12.16	2.27	5.35	0.003
EO Concentration	0.02	0.013	2.05	0.096
Parameter	Lower 95%	Upper 95%		
Constant	6.323	18		
EO Concentration	-0.007	0.063		

F. solani

Parameter	Estimate	s. e.	t(5)	t pr.
Constant	7.35	0.929	7.92	<0.001
EO Concentration	0.020	0.005	3.72	0.014
Parameter	Lower 95%	Upper 95%		
Constant	4.963	9.737		
EO Concentration	0.006	0.035		

Appendix C: Analysis of variance (ANOVA) on the inhibition of *Tagetes minuta* essential oils and Enrich BM® on three test bacteria after 24 and 48 hours incubation

Source of variation	d. f.	s. s.	m. s.	v. r.	F pr.
Rep					
Bacteria	2	648.37	324.18	199.97	<0.001
24_48hrs	1	0.84	0.84	0.52	0.479
Essential_EnrichBM	1	10.56	10.56	6.52	0.018
Bacteria-24_48hrs	2	9.43	4.71	2.91	0.076
Bacteria-Essential_EnrichBM	2	688.79	344.39	212.43	<0.001
24_48hrs-Essential_EnrichBM	1	0.84	0.84	0.52	0.479
Bacteria-24_48hrs-Essential_EnrichBM	2	30.51	15.25	9.41	0.001
Residual	22	35.66	1.62		
Total	35	1426.18			

Appendix D: Linear regression analysis of inhibition zone diameters against the concentration of *Tagetes minuta* essential oil for the test bacteria.

Pseudomonas savastanoi pv. *phaseolicola*

Parameter	Estimate	s. e.	t(5)	t pr.
Constant	7.24	0.96	7.51	<0.001
EO Concentration	0.04	0.005	7.15	<0.001
Parameter	Lower 95%	Upper 95%		
Constant	4.76	9.73		
EO Concentration	0.02	0.05		

Xanthomonas axonopodis pv. *manihotis*

Parameter	Estimate	s. e.	t(5)	t pr.
Constant	6.56	0.59	10.99	<0.001
EO Concentration	0.01	0.003	5.18	0.004
Parameter	Lower 95%	Upper 95%		
Constant	5.027	8.097		
EO Concentration	0.009	0.027		

Xanthomonas axonopodis pv. *phaseoli*

Parameter	estimate	s. e.	t(5)	t pr.
Constant	6.65	0.55	12.03	<0.001
EO Concentration	0.03	0.003	9.17	<0.001
Parameter	Lower 95%	Upper 95%		
Constant	5.23	8.07		
EO Concentration	0.02	0.03		

Appendix E: Mass spectral fragmentation pattern of compounds identified in *Tagetes minuta* essential oils

No	Retention time (min)	Compound name	Major fragmentation ions and their masses
1	10.6	Sabinene	m/z 136[25% M^+], 121[5% ,M-15], 93[100%,M-43], 77[100%,M-59]
2	11.2	α-Phellandrene	m/z 136[25% M^+], 121[5% ,M-15], 93[100%,M-43], 77[27%,M-59]
3	11.7	Limonene	m/z 136[25% M^+], 121[25% ,M-15], 93[50%,M-43], 68[100%,M-68]
4	11.8	(Z)-β- Ocimene	m/z 136[5% M^+], 121[20% ,M-15], 93[100%,M-43]
5	12.0	(E)-β- Ocimene	m/z 136[1% M^+], 121[20% ,M-15], 93[100%,M-43]
6	12.1	Dihydrotagetone	m/z 154[1% M^+], 97[20% ,M-57], 85[100%,M-69]
7	12.8	Unknown	m/z 136[50% M^+], 121[100% ,M-15], 105[50%,M-43]
8	13.4	Allo-Ocimene	m/z 136[50% M^+], 121[100% ,M-15], 105[40%,M-43]
9	13.5	Unknown	m/z 152[10% M^+], 137[100% ,M-15], 121[20%,M-31]
10	13.6	Unknown	m/z 152[1% M^+], 136[50% ,M-15], 121[100%,M-31]
11	13.7	(E)-Tagetone	m/z 152[10% M^+], 95[100% ,M-57], 109[20%,M-43]
12	13.8	(Z)-Tagetone	m/z 152[40% M^+], 95[100% ,M-57], 109[40%,M-43]
13	15.0	(Z)- Ocimenone	m/z 150[50% M^+], 135[100% ,M-15], 107[40%,M-43]
14	15.1	(E)- Ocimenone	m/z 150[100% M^+], 135[100% ,M-15], 107[60%,M-43]
15	15.6	Unknown	m/z 150[30% M^+], 135[50% ,M-15], 82[100%,M-70]
16	16.6	Elixene	m/z 204[3% M^+], 121[100% ,M-83], 93[80%,M-111]
17	16.7	Silphiperfol-6-ene	m/z 204[98% M^+], 189[100% ,M-83], 93[80%,M-111]
18	17.7	(E)-Caryophyllene	m/z 204[5% M^+], 189[90% ,M-15], 175[100%,M-29]
19	18.2	α-Humulene	m/z 204[5% M^+], 147[10% ,M-57], 93[100%,M-111], 121[27%,M-83]
20	18.7	Bicyclogermacrene	m/z 204[5% M^+], 161[7% ,M-43], 93[100%,M-111], 121[92%,M-83]

m/z - Mass-to-charge ratio